University of Alberta

Prevention of Oxidative Injury in Islet Isolation

by

José Gabriel Avila Calles

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Dedication

To Marietta, whose love, patience and hopes have guided us through this path in order to fulfill our dreams together. To José, Anna and Rebecca for being part of those dreams. I dedicate it also to my father Hermilo, who still lives in me for every single thing I do in life, and to my mother Yreima, for all her care and unconditional support.

Abstract

Islet transplantation has emerged as a plausible therapy for the treatment of type 1 diabetes. However, islets are faced with various types of stress related to the isolation and transplantation procedure. Variable periods of ischemia and oxidative stress can affect the number and function of islets recovered, frequently having to pool islets from more than one donor to achieve normoglycemia in one recipient.

Our rodent model of pancreatic warm ischemia showed that ischemia during preservation and islet isolation increased oxidative stress, damaging islets and affecting isolation outcomes. Our studies further revealed that by adding an antioxidant precursor (L-Glutamine) to the standard intraductal pancreas perfusate together with the collagenase enzyme, we were able to increase the levels of Glutathione in islets. This showed improvement in islet yields, viability and function after isolation and transplantation, compared to the standard intraductal flush. Furthermore, when applying this therapy in the clinical setting, isolation outcomes were improved by glutamine treatment and oxidative stress was decreased in glutamine-treated islets leading to lower oxidative injury and apoptosis than the control. As in the rodent studies, *in vivo* islet function was also improved after transplantation into diabetic athymic mice, rendering a higher percentage of transplanted mice normoglycemic in a shorter period of time than the control.

Ischemia produces damage of mitochondrial structures and precedes the occurrence of oxidative stress by the introduction of O_2, an entity known as reperfusion injury. We tested the effect of pancreatic intraductal administration of a hemoglobin-based O_2 carrier (PolyHeme) loaded with O_2, after 30 min warm ischemia. Viability, *in vitro* and *in vivo* function of islets were significantly improved by PolyHeme pre-treatment. Mitochondrial integrity and function were also superior in treated islets shown by improved mitochondrial membrane potential and increased fluorescent intensity

evenly distributed around the nuclei. In our study, the introduction of O_2 by PolyHeme did not increase oxidative stress or damage to the islets.

The intraductal administration of Glutamine and Hemoglobin O_2 carriers (PolyHeme) as a simple addition to current standard isolation techniques provides protection of pancreatic islets from oxidative stress and ischemia, improving islet isolation outcomes and islet function after transplantation.

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LIST OF ABBREVIATIONS:

2,3 DPG: 2,3 Diphosphoglycerate

ADP: Adenosine Diphosphate

AGEs: Advanced Glycation Endproducts

ALP: Alkaline Phosphatase

AMP: Adenosine 5'-Monophosphate

ANOVA: Analysis of Variance

ANT: Adenine Nucleotide Translocator

ATP: Adenosine Triphosphate

BMI: Body Max Index

cAMP: Cyclic Adenosine Monophosphate

CIT: Cold Ischemia Time

COHb: Carboxyhemoglobin

CoQ: Coenzyme Q

COX: Cytochrome C Oxidase

CuSOD: Copper Superoxide Dismutase

Cyt c: Cytochrome C

DCCT: Diabetes Control and Complications Trial

DMSO: Dimethyl Sulfoxide

EC: Energy Charge

EDTA: Ethylediamine Tetraacetic Acid

EGF: Epidermal Growth Factor

EIN: Equivalent Islet Numbers

ERK: Extracellular Signal-Regulated Kinase

ERSD: End-stage Renal Disease

Fe^{2+}: Ferrous Iron

Fe^{3+}: Ferric Iron

FI: Freshly Isolated

FPG: Fasting Plasma Glucose

GDM: Gestational Diabetes Mellitus

GSH: Reduced Glutathione

GSH_{px}: Glutathione Peroxidase

GSSG: Oxidized Glutathione

H_2O_2: Hydrogen peroxide

HBOCs: Hemogoblin-based O_2 Carriers

HPN: Home Parental Nutrition

HX: Hypoxanthine

IBMIR: Immediate Blood Mediated Inflammatory Response

IDDM: Insulin-Dependent Diabetes Mellitus

IE: Islet Equivalent

IGT: Impaired Glucose Tolerance

INOS: Inducible NOS

IPG/AST: Intraperitoneal Glucose/Arginine Stimulation Test

IPGTT: Intraperitoneal Glucose Tolerance Test

IPTR: International Pancreas Transplant Registry

I/R: Ischemia/reperfusion

ITN: Immune Tolerance Network

ITR: Islet Transplant Registry

JNK: C-Jun Amino-Terminal Kinase

MDA: Malondialdehyde

MAPK: Mitogen-Activated Protein Kinase

MetHb: Methemoglobin

MHC: Major Histocompatibility Complex

MnSOD: Manganese Superoxide

NADH: Nicotinamide Adenine Dinucleoide

NIDDM: Non-Insulin-dependent diabetes mellitus

NO: Nitric Oxide

NO•: Nitric Oxide

NOS: Nitric Oxide Synthase

O_2: Oxygen

O_2•: Superoxide Anions

OH•: Hydroxyl Radicals

OFR: Oxygen Free Radical

$ONOO^-$: Peroxynitrite Anion

PAK: Pancreas After Kidneys Transplant

PARP: Poly(ADP-ribose) Polymerase

PBS: Phosphate-Buffered-Saline

PCO_2: Carbon Dioxide Tension

PFC: Perfluocarbons

PI(3): Phosphoinositide 3

PO_2: Partial Pressure of O_2

PTA: Pancreas Transplant Alone

•Q-: Semiquinone Anion Species

RBC: Red Blood Cell

RhGH: Recombinant Human Growth Hormone

RIN m5F: Rat Insulinoma Cell Line

RL: Ringer's Lactate

RNS: Reactive Nitrogen Species

ROS: Reactive Oxygen Species

SE: Standard Error

SOD: Superoxide Dismutase

SPK: Simultaneous Pancreas-Kidney Transplant

TLM: Two Layer Method

UNOS: United Network for Organ Sharing

UW: University of Wisconsin

Chapter 1

Introduction

Part 1) Diabetes Mellitus

History of Diabetes

Diabetes mellitus comprises a group of common metabolic disorders that share the phenotype of hyperglycemia. Diabetes is a chronic disease caused by inherited and/or acquired deficiency in production of insulin by the pancreas, or by ineffectiveness of the insulin produced, resulting in a disordered carbohydrate, lipid and protein metabolism. Such deficiency results in increased concentration of glucose in blood, which in turn leads to damage of many of the body's systems, especially the blood vessels and nerves. Diabetes has been recognized for thousands of years. In the Greece of the 2nd century, the disease was already viewed as "a melting down of the flesh and limbs into the urine" (1). In the 19th century, scientists, especially in Europe, were beginning to concentrate on the pancreas as the "defective" organ (1). The French physician Lancereaux is generally credited with making the distinction between fat and thin diabetes: "diabete gras and diabete maigre" (2). After the discovery of insulin in 1921, diabetes entered a new era (3), people with diabetes did not die, and many initially thought that diabetes had been cured. More into recent times, diabetes by the mid-1990's, was already seen as a major international health problem (4) and a costly clinical public health disorder (5).

Classification and Diagnosis

The first test used to distinguish between the two main forms of diabetes was the response to insulin, when Wilhelm Falta and other investigators in Vienna drew attention to the existence of insulin-sensitive and –resistant, forms of diabetes (2). However, the World Health Organization and American Diabetes Association classifications are currently shifted to a system that defines diabetes in terms of pathogenesis. The four main groups are: 1) Insulin-dependent diabetes mellitus (IDDM), which has been replaced by Type 1 (immune mediated or idiopathic β-cell dysfunction leading to absolute insulin deficiency). These patients have β-cell destruction, which is usually immune-mediated; most develop absolute insulin deficiency and are ketosis-prone, 2) non-insulin-dependent diabetes mellitus (NIDDM) has been replaced by Type 2 diabetes, which encompasses the most prevalent form of the disease. Most patients with Type 2 diabetes exhibit insulin resistance and ultimately develop a concomitant insulin secretory defect, 3) the third class includes genetic defects in insulin secretion and action, diseases of the exocrine pancreas, endocrinopathies that induce hyperglycemia, drug-induced forms of diabetes, infectious causes, and genetic syndromes that are associated with an increased incidence of diabetes, and 4) gestational diabetes mellitus (GDM), which remains a separate class as before (6, 7).

New criteria for the diagnosis of diabetes reflect an appreciation of the fact that the oral glucose tolerance test is rarely used in practice. Using the revised criteria, there are three ways to diagnose diabetes mellitus: 1) random, or casual plasma glucose ≥200 mg/dL (11.1 mmol/L), associated with symptoms (polyuria, polydipsia, unexplained weight loss); 2) fasting plasma glucose (FPG) ≥126 mg/dL (7.0 mmol/L); 3) 2-h glucose ≥200 mg/dL (11.1 mmol/L) after a 75-g glucose load. Any of these criteria are sufficient for diagnosis, but each should be confirmed on a separate day (8, 9).

The decrease in FPG threshold from 140 mg/dL to 126 mg/dL is the major change in these diagnostic criteria. The previous FPG level of 140 mg/dL was not as sensitive as the new criteria potentially missing a group of patients, which remained undiagnosed (8). A category of impaired glucose tolerance (IGT) is retained for individuals with fasting plasma glucose ≥110 mg/dL but <126 mg/dL. Patients in this group are at increased risk for the development of diabetes and cardiovascular disease (9, 10).

Etiology

Type 1 diabetes mellitus is perceived as a chronic autoimmune disease with a sub-clinical prodrome characterized by selective loss of insulin producing beta cells in the pancreatic islets in genetically predisposed subjects (11). Less than 10% of those with increased genetic susceptibility progress to clinical disease suggesting a strong environmental modification of the prediabetic process (11). Various exogenous triggers, such as certain dietary factors and viruses, are thought to induce the autoimmune process leading in some individuals to extensive β-cell destruction and ultimately to the clinical manifestation of type 1 diabetes (12). An interesting environmental factor is milk intake, which is discussed as possibly playing a pathogenic role. Geographical and temporal relations between type 1 diabetes prevalence and Cow's milk consumption have been found in ecological studies (13).

The etiology of type 2 diabetes is also multifactorial relating to dietary factors (obesity) and sedentary lifestyle, which seem to be among the strongest risk factors (14). Type 2 diabetes is genetically heterogeneous (multiple different genes involved) and with molecular techniques like restriction fragment length polymorphisms and genomic sequencing, various loci have been evaluated (15).

Epidemiology.

Diabetes is now a global health problem, and populations of developing countries, minority groups, and disadvantaged communities in industrialized countries now face the greatest risk (16).

An estimated 177 million people suffer from diabetes mellitus type 1 and type 2 worldwide (17), and that number may well double by the year 2025 (18). Between 1995 and 2025 the World Health Organization projects that the number of adult population affected by diabetes mellitus in developing countries will grow by 170%, from 84 to 228 million people. Worldwide, a rise is projected to make a total of 300 million people. Much of this increase will occur in developing countries and will be due to population ageing, unhealthy diets, obesity and sedentary lifestyle (19).

The incidence of type 1 diabetes among children age <19 years in the U.S. is an estimated 18.2 per 100,000/year, that is 13,171 new cases each year. In adults an estimated 16,542 cases of type 1 diabetes arise each year in persons age >20 years in the U.S. (20, 21). In Montreal, Canada, there are large differences among ethnic groups. In contrast, Japan has a very homogeneous population and shows little variation among studies (22). Of the 16 million people with diabetes in North America, an estimated 1.7 million are type 1 and the rest are patients with type 2 diabetes (23).

Costs

Because of its chronic nature, the severity of its complications and the means required to control them, diabetes is a costly disease, not only for the affected individual and his/her family, but also for the health authorities. The total health costs of a person with diabetes in the USA are several times more than for people without the condition. The per capita annual costs of health care for people with diabetes rose from $10,071 in 1997 to $13,243 in 2002, an increase of more than 30%. In contrast, health care costs for people without diabetes amounted to $2,560 in 2002. Intangible costs also have a great impact on lives of patients and their families, and are the most difficult to quantify. The direct costs of diabetes include medical care, drugs, insulin and other supplies, representing a burden to society of approximately $92 billion estimated in 2002. The indirect costs come from the number of diabetic patients that may not be able to continue working or work as effectively as they could before the onset of their condition, which represent approximately $40 billion. Sickness absence, disability, premature retirement or premature mortality can cause loss of productivity (17). Altogether, indirect and direct costs were calculated to be $132 billion in 2002 (24). This represents 19% of total personal health care expenditures in the U.S. However, diagnosed diabetes patients account for only 4.2% of the total U.S. population.

Part 2) Endocrine Replacement Therapies

The administration of exogenous insulin has been the primary treatment for type-1 diabetes since the discovery of insulin in 1921 by Banting and Best (25). Although the survival rates for diabetes have increased substantially, exogenous insulin therapy does not represent a cure for this disease. Moreover, data from the Diabetes Control and Complications Trial (DCCT) in 1993 showed that intensive insulin therapy could significantly reduce the risk of neurovascular complications, (50% reduction in eye, nerve, and kidney complications), but increases the risk of hypoglycemia by three times (26).

Islet Transplantation

The idea of islet transplantation is not entirely new; it has been around since the late 1800's, when Watson Williams attempted the transplantation of few pieces of freshly slaughtered sheep pancreas subcutaneously in a 15 year old end-stage diabetic boy, at the University of Bristol (UK) (27). Frederick Pybus, later in 1916 described patients transplanted subcutaneously with human sliced pancreata (28). Experimental research had already started in 1911 with Bensley (29) by staining of pancreatic islets, followed in 1964 by Hellerstrom (30), who used free-hand micro-dissection as a method of obtaining islets. In 1965 Moskalewski (31) was able to isolate pancreatic islets from guinea pigs by collagenase digestion (enzymatic method), followed by Lacy and Kostianovski in 1967 (32) with the isolation of a large amount of rat islets using intraductal collagenase digestion and purification from the exocrine tissue. The real era of islet transplantation started with Ballinger and Lacy (33) in 1972 with transplantation of rat islets, followed by Reckard and Barker in 1973 who effectively cured diabetes in animals for the first time (34). The first series of clinical islet allografts were performed in 1977 by Najarian and Sutherland (35), but the modern era of islet transplantation started in 1988, after Camillo Ricordi and collaborators developed a new method for pancreas dissociation and islet purification (36).

Despite improvements obtained in islet isolation techniques, the results of islet transplantation before the year 2000 continued to be dismal. In reports from the International Islet Transplant Registry (ITR) in the period of 1990-1995 indicate that only 6% of type 1 diabetic islet recipients achieved exogenous insulin-independence 1 year post-transplantation (37). In the 1990-1998 era, the percentage of patients who had developed insulin-independence for more than 7 days was 12% (38). In 1999, the ITR report (39) concluded that establishment of insulin independence after islet transplantation was associated with the following factors: 1) pancreatic preservation times (less or equal to 8 h), 2) islet mass transplanted is adjusted to body weight (6,000 islet equivalents (IE) per kilogram of body weight, 3) intrahepatic transplantation, and 4) induction with monoclonal or polyclonal T-cell antibodies. Taking these factors into consideration, the turning point in modern islet transplantation was marked by the report of Shapiro and collaborators in the year 2000 (40), when they achieved 100% success (insulin-independence) one year post transplantation in a series of 7 patients performed at the University of Alberta in Edmonton, Canada.

The Edmonton Protocol

The Edmonton Protocol based their success in 1) a new steroid-free immunosuppressive therapy combination formed by Sirolimus, a low dose calcineurin inhibitor (Tacrolimus), and induction with an anti-IL2-receptor antibody (Daclizumab), 2) sufficient number of high-quality human islets (over 10,000 IE/kg body weight of recipient), and 3) recipient selection; patients with brittle type 1 diabetes without renal failure.

The potential causes of failure of islet transplants had been determined and included failure of initial engraftment, inflammatory response at the transplant site, allo- or autoimmune response, and immunosuppressive drug-induced β-cell toxicity (41). The Edmonton Protocol not only addressed these issues, but further optimized islet function by controlled delivery of a purified low-endotoxin collagenase enzyme, immediate graft processing, and transplantation of a total average of 800,000 IE (just over 11,000 IE per kg recipient body weight) into the liver via the portal vein (40). The experience of the Edmonton Trial has been reproduced in other centers through the Immune Tolerance Network (ITN) Trial, which started in the year 2000 and included Centers in Edmonton, Minneapolis, Miami, Seattle, St. Louis, Boston, Geneva, Giessen and Milan. The objectives in the creation of this trial were to replicate the Edmonton Protocol at multiple sites, provide a base of qualified islet centers for future ITN tolerance trials, and to explore mechanisms of islet acceptance/rejection.

The protocol has been difficult to replicate with success in some of the centers, although, three of the most experienced centers achieved a 90% insulin-free rate after 1 year (42). In fact, 5 of the first 13 ITN patients (38%) achieved insulin-independence with a single donor islet transplant, and single donor success was as high as 75% at one of the centers (42). Preliminary analysis suggests that the two most significant factors affecting clinical success are 1) the center's skills in preparation of high-quality, high-yield islets following identical isolation protocol, and 2) the center's ability to maintain the recipient's immunosuppressant levels within a specified target range after transplantation (43). Despite these encouraging reports, the results of islet transplantation among different centers continue to be variable, and show limitations to this procedure (44). To date, after 5 years follow-up of patients in the Edmonton Protocol, we have learned that most patients have reverted to using some insulin, although C-peptide secretion (regarded as islet graft survival) has been maintained in the recipients over this period, which is still beneficial for the internal regulation of blood glucose levels (glucose homeostasis) (45).

Different Types of Islet Transplants

There are potential patients to consider that will benefit from an islet transplantation procedure (46):

Allotransplantation: There are two major categories in this group, they are 1) Patients with type 1 diabetes who need a kidney graft: Kidney transplant recipients will receive immunosuppressant therapy for this operation and the addition of an islet transplantation would only represent an extra step; 2) Patients with type 1 diabetes who have previously received a kidney: In order to protect the transplanted kidney when already systemic complications are developing or when a normal glycemic regulation is difficult to maintain. Two additional situations can be considered: Patients with type 1 diabetes where a previous whole pancreas transplant is lost by rejection or surgical complications, and in some specialized centers, patients with localized abdominal tumors undergo upper abdominal exenteration followed by liver and islet transplantation (47).

Autotransplantation: This type of transplantation has been successful in non-diabetic patients with chronic painful pancreatitis (48, 49) or pancreatic tumors which need to be resected (50). In patients with chronic pancreatitis abdominal pain is finally treated by total pancreatectomy and islets can be isolated from their native pancreas in a few hours and then infused back into their portal vein. Insulin responses to oral and intravenous glucose, as well as to intravenous arginine, have been reported intact in these patients for over 7 years post-transplantation (51). The isolation of islets from these patients is difficult due to the fibrotic consistency of the pancreas as a result of chronic pancreatitis, however, there are several advantages over allo-islet transplantation: 1) The recipients do not need immunosuppressive therapy, since the graft is coming from the same recipient; 2) islets do not have to undergo purification during the isolation, where approximately 20-30% of islets are lost; 3) the number of islets needed to achieve insulin-independence is less than allotransplant recipients; and 4) autograft recipients have not previously undergone an autoimmune attack that resulted in diabetes. The decision of when to perform the pancreatic resection in these patients is challenging, since it should be considered that there is the potential for transforming a patient with a decent blood glucose regulation, into an insulin-dependent diabetic. However, the decision is usually driven by pain and frequency of visits to the hospital because of pain or complications. This therapy would be justified when these complications outweigh the risks of the procedure (52).

Living Donor Islet Transplantation: A new category of islet transplantation has developed; living donor islet transplantation has been proven successful in the hands of a specialized team (53). This report demonstrated the achievement of insulin-independence in a living islet transplant recipient with donation from Mother-to-Daughter. Five months after the procedure, the recipient continues to be insulin-free and without hypoglycemic unawareness. It is noteworthy to mention that this transplant was performed in a non-autoimmune patient. However, with the percentage of variability in the results of islet isolation, living donor islet transplantation remains controversial (54). Nevertheless, there are potential benefits for living donation: 1) the achievement of better HLA matching, 2) the possibility of prospective cross match, 3) lower rates of post-transplant infections and malignancies due to milder immunosuppression protocols, 4) a reduced period of organ preservation and ischemia, and 5) alleviation of the current organ shortage and reduced waiting list time for recipients. On the other hand, these advantages for the recipients may be difficult to justify because of the obvious disadvantages for the donor.

Pancreas Transplantation

Pancreas Transplantation was first used for the treatment of type 1 diabetes in humans in 1966 (55). Because of low survival rates for graft and patients, improvements had to be made in immunosuppressive regimens and new surgical techniques (56). It was not until after a decrease in technical and immunological failure that the number of transplants and survival rates steadily increased each year from 40% patient survival and 5% graft survival in the 1966-1977 era, to 95% and over 85% respectively for 2003 (57). Pancreas transplants are done in three different categories of diabetic recipients, classified according to the kidney function status: 1) Simultaneous pancreas-kidney transplants (SPK) in uremic patients or patients with end-stage renal disease (ERSD); 2) Pancreas after (previous) kidney transplants (PAK) in post uremic patients; and 3) a pancreas transplant alone (PTA) in non uremic patients (58). The vast majority of deceased donor pancreas transplants (>75%), according to the United Network for Organ Sharing (UNOS) and the International Pancreas Transplant Registry (IPTR) data, are in SPK category, with the aim of establishing an insulin-independent as well as a dialysis-free state (57). It is shown that with SPK transplants, there is the possibility to arrest the progress of ongoing secondary complications, and to improve the quality of life (41). Survival of pancreas grafts when transplanted alone (PTA) is lower than SPK (59), but this may be due to the lack of a marker for pancreas rejection that is as sensitive as serum creatinine is as a marker for kidney transplant rejection (41). What this means is that since in SPK, rejection of the kidney would mean most likely rejection of the pancreas graft as well, the immunosuppressive therapy can be changed or adjusted in time to salvage the graft.

Pancreas transplantation offers remarkable improvement in quality of life for diabetic patients who have end-stage diabetic nephropathy requiring renal transplantation (60), reduces cardiovascular risk factors (61), and improves overall survival in uremic diabetic patients with ESRD (62). However, this combined procedure is associated with significant morbidity and mortality compared to islet transplantation (63). Most of the complications for pancreas transplants are related to 1) Technical complications and surgical technique (>10%), 2) Immunological, due to rejection (<10%), 3) Recurrent diabetes, 4) Post-transplant lymphoproliferative disorders, and 5) Drug toxicity (64). Although the magnitude of the surgery is less for islet than for pancreas transplantation, the latter is more effective in terms of endocrine function (insulin-independence) and number of donors required (one) to achieve insulin-independence (65).

Islet Transplantation vs. Whole Pancreas Transplantation

A low-level invasive procedure of islet transplantation would be expected to be safer and much less costly than whole pancreas transplantation; however, this procedure also requires long-term immunosuppression. Since the goal of any pancreas or islet transplantation is the achievement of excellent glycemic control with minimal risks, islet transplantation offers the hope that if performed earlier it will result in excellent glucose control and prevent long-term complications (66).

In a study that compared efficacy and costs of pancreas vs. islet transplants reported by Frank and collaborators from the University of Pennsylvania (67), it was shown that in the short-term follow-up, pancreas and islet transplants were equally effective; however, insulin independence was not maintained over an extended period of time in the islet transplant patients. Maintenance of insulin independence was better for whole pancreas grafts within 2 months and became increasingly pronounced over time in this study. In 26 whole pancreas transplant patients insulin independence remained at 100% at 2 years after transplantation. From 11 islet recipients, insulin-free survival rate was 100% at 1 month post-transplantation, 91% at 3 months, and down to 51% at 12 months. A decrease in islet graft function was also observed by the Edmonton group, whose results appear to be the best so far with 80% insulin-independence for 1 year, 70% for 2 years and 60% for 3 years (66, 68). The Edmonton group has recently shown that after 5 years follow-up insulin-independence has decreased to 10%. Nonetheless, in the Pennsylvania study, when graft survival (C-peptide secretion) was compared between pancreas and islet transplants, islet graft survival more nearly approximated that of pancreas grafts (67). These results relate to those found in Edmonton, which show 80% graft survival (determined by C-peptide secretion) after 5 years (45).

Maintaining insulin-independence by islet transplants has been unexpectedly found to be more expensive than pancreas transplants, mostly related to the frequent need of multiple pancreata for one islet recipient. In addition, if the organs that were processed, but not transplanted are considered, the costs are further increased (67).

The idea of pancreas and islet transplant programs coexisting in a complementary way in the same institution is possible, since it has been shown that these programs may have very small competition for organs (69). This is based on patient selection criteria for each procedure and the amount of pancreas offers in an Institution. Until the limited availability and survival of islets can be increased by improved isolation techniques and a more effective immunosuppression, pancreas and islet programs should continue to coexist in a complementary way (67).

Factors Influencing Islet Availability

One of the main goals currently in islet transplantation is the achievement of insulin independence with islets from 1 donor pancreas, and this requires a consistent, successful islet isolation (70). Although islet isolation has progressively improved, islet yields are still highly variable. Poor donor selection (donor characteristics), conditions of pancreas procurement and storage, and the inconsistency of enzyme blends used for digestion are some of the parameters that contribute to this variability (71-73).

Pancreatic Islet Isolation

The procedure of islet isolation is conformed by three main steps:

Pancreas distention with Collagenase enzyme: The dissociation of the endocrine pancreas (islets) from the exocrine tissue (acinar and ductal cells) is performed by destruction of the collagen bonds between these two components. The enzyme is based on the activity of a protease from Clostridium histolyticum. It is distributed through the whole pancreatic tissue through the main pancreatic duct. This step is performed at cold temperatures (approx. 4°C).

Pancreas digestion: The digestion step of the isolation comprises a mechanical dissociation (shaking of the pancreas in the Ricordi chamber with steel marbles) and a chemical dissociation (enzyme activity at 37°C). The digestion is stopped by dilution of the preparation with cold solutions and quenching of the enzyme with concentrated albumin solutions.

Islet Purification: After the washing and collection of the tissue, the islets are physically separated in the preparation from the exocrine tissue using a density gradient (Ficoll), by centrifugation.

A Swiss report has determined factors affecting yields for a successful isolation (74). Among donor-related factors they found age over 20 years old, body mass index (BMI) over 25 kg/m^2, warm ischemia (from cross-clamp to pancreas excision) less than 30 minutes and cold ischemia less than 8 hours to be positively associated with successful outcomes. Matsumoto and collaborators have pointed out additional "risk factors" in the donor that interfere with a successful islet isolation (75), including are: peak transaminases levels two times higher than the normal value, peak creatinine levels more than 1.5 times higher than the normal value, length of hospitalization for more than 96 hours, norepinephrine usage for stabilization of cardiac function or cardiac arrest, and total pancreas preservation time in UW (cold ischemia) more than 16 hours. The window of length in the period of ischemia has been broadened thanks to the use of the Two Layer Method (TLM) with perfluorocarbons (PFC), which will be explained later.

In addition, now that the intra-lot variability of the purified collagenase used for human islet isolation has been finally proven, it can explain historical experiences of notable failures with prime donor pancreata, and surprising successes with marginal donors (76). Efforts are ongoing in the search of a predictive system for results of the isolation procedure (77, 78).

Pancreas Procurement

Organ procurement is a critical step that can influence the outcome of any transplantation procedure. In a multi-organ cadaveric donor, there is a “race against time” once the cross clamp of the aorta has been performed and blood supply to the organs is halted. At this point, there are several procedures that take place in order to protect organs from the deleterious effects of ischemia, characterized by the cold flush of preservation solutions through their vasculature and the decrease in core temperatures by the addition of sterile ice. Those more susceptible to the effects of ischemia are procured first (heart and lungs), followed by the abdominal organs, of which usually liver precedes the excision of pancreas and kidneys.

In the case of pancreas procurement for islet transplantation, it has been demonstrated that the procurement technique and the maintenance of a low temperature are critical to the subsequent islet yield and function (79, 80). Better outcomes in islet isolation have also been shown from pancreata procured even before liver excision (80). Careful dissection of the pancreas and its vasculature preventing any parenchymal injury or vascular disruption to the organ are also important factors that influence the quality of the pancreas for islet isolation.

It is possible that pancreatic tissue is at risk of exposure to a period of warm ischemia during procurement. This period could be variable, depending in part on the surgeons and the protocols at different institutions where organs are being recovered, pointing out the importance of strategies targeted not only to the prevention of warm ischemia, but also the recovery of the tissue in case of exposure.

Part 3) Organ Preservation

Effects of Ischemia

In organ transplantation, the interruption of blood flow is a necessary action that results in a decrease of oxygen and nutrient delivery to the cells (ischemia) with subsequent cell damage. The role of cold storage in organ preservation is the maintenance of viable tissue during ischemia by reduction of cell metabolism and nutrient demand.

In organ preservation, ischemia (warm and cold) represents the main target to overcome. One of the predominant effects of ischemia is inadequate energy production (adenosine 5'-triphosphate or ATP) (81). There are two modes of providing ATP: one of them is oxidative phosphorylation or mitochondrial respiration, where most of the ATP is synthesized (Figure 1-1). The other one is glycolysis, which produces oxidation to ATP from ADP and inorganic phosphate (Pi) (Cytosolic).

ATP production halts during ischemia, shifting aerobic metabolism to anaerobic by activating the glycolysis pathway, which may be the only means of producing ATP in the absence of O_2. Glycolysis produces a limited amount of ATP (2/molecule of glucose) compared to oxidative phosphorylation (36/molecule of glucose), but this comes at a metabolic cost. Glycolysis may even enhance the damage by further reducing pH through the production of protons and lactic acid accumulation (82). Cell acidosis from anaerobic metabolism and the depletion of ATP production results in alteration of membrane enzymes, especially Na^+/K^+ and Ca^{2+}/Mg^+ ATP-dependent pumps. With the cessation of the Na^+/K^+ ATPase, intracelullar Na^+ rises and K^+ falls, as the membrane potential is reduced it causes voltage-dependent Ca^{2+} channels to open and an increased influx of calcium ion. Higher intracellular concentrations of Ca^{2+} activate membrane phospholipases, which catalyze hydrolysis of cell-membrane and organelle phospholipids. Activation of mitochondrial phospholipases disrupt oxidative phosphorylation, further reducing ATP production. With depolarization, Cl^- also enters the cell, so NaCl is introduced into the cell bringing water with it and causing cell swelling, which can continue onto cell lysis, and death. Moreover, the oncotic pressure of the internal proteins also pulls water into the cells resulting in additional swelling (83). Up to a certain point, the changes can be reversible, but once mitochondria condense and lysosomal membranes are damaged, enzymes are released, causing structural damage to proteins and cell death.

Ca^{2+} also plays an important roll in the regulation of key enzymes responsible for mitochondrial respiration, ATP transport, and regulation of ionic transport and membrane potential (84). Cytosolic Ca^{2+} can restore or preserve the enzymatic reactions needed for the integrity of the hypothermic ischemic cell, but also inhibits other enzymatic systems such as pyruvate kinase. Ca^{2+} may exert its functions by activation and deactivation of other intracellular messengers, including regulation of adenyl cyclase and phospohodiesterase (85). During ischemia and specifically during hypothermic storage the enzymatic system (Ca^{2+}/Mg^{2+} pump) primarily responsible for the efflux of Ca^{2+} across plasma membranes are deactivated, causing a massive influx of calcium into the cytosol with the deterioration of cellular function (84).

Effect of Hypothermia

Hypothermia is an effort by cold solutions to reduce the rate of energy utilization (ion pump, contraction) in order to minimize the rate of energy production (ATP synthesis) (81). This concept is explained by the Q_{10} effect, which is the ratio of the rate of a reaction at one temperature divided by the rate of the same reaction at a temperature 10°C less. Hence, most enzyme systems show a 1.5- to 2.0-fold decrease in activity for every 10°C decrease in temperature (86). Membrane functions have temperature related properties; the components of cell membranes are normally and randomly distributed in the plane of the membrane, all in dynamic equilibrium. Lowering the temperature of the system can upset this equilibrium by reducing membrane fluidity and inducing separations between lipids and proteins (87). Nevertheless, by reducing the rate at which substrates are metabolized, cooling makes cells less dependent on their supply (slowing of chemical reactions and reduced demand for O_2), which could be beneficial. The problem lies in the fact that especially the Na^{+}/K^{+} pump is slowed down by cooling, resulting in a similar effect produced by anoxia, but by a different mechanism. At low temperatures, the pump is unable to utilize ATP, resulting invariably in cell swelling and damage (81). Preservation solutions have generally been formulated with high potassium content such as Collins, Euro-Collins and University of Wisconsin (UW) solution to counteract the loss of ions gradients across the membrane. To prevent cell swelling, the addition of substances impermeant to the cell with osmotic pressure (110-140 mOsm/kg osmotic force) is used for cold storage. UW solution contains raffinose and lactobionic acid, which are impermeable to the endothelium and thus maintains intravascular oncotic pressure, preventing water from entering the cell (88).

Ischemia/Reperfusion Injury

The biochemical and structural alterations produced during preservation are precursors to the more extensive morphological and functional damage generated upon re-introduction of oxygen, a phenomenon known as ischemia/reperfusion (I/R) injury. I/R injury involves a complex interaction among oxygen free radicals, neutrophils, cytokines, and other inflammatory mediators, with resultant direct injury at the cellular level. Clinically, I/R injury is a major cause of graft dysfunction or failure, and other complications that can ultimately lead to death.

Oxidative Stress

Oxidative stress is a condition in which the amount of strong oxidants produced is greater than those that can be scavenged by antioxidative mechanisms. In the course of normal metabolism, oxidizing equivalents or reactive oxygen species (ROS) are generated when oxygen is partially reduced as electrons leak out of the electron transport chain during respiration in mitochondria (89). Accumulating evidence implicates ROS as specific signaling molecules under both physiological and pathophysiological conditions (90). The generation of ROS, with certain boundaries, is essential to maintain homeostasis. For example, ROS generation by phagocytic cells constitutes an essential host defense mechanism necessary to combat infection (90). Under certain situations of metabolic stress, mitochondrial-derived oxidants seem to function as signaling molecules and regardless of how they are generated, a rise in intracellular oxidant levels has two potentially important effects: 1) damage to various cell components and 2) triggering of the activation of specific signaling pathways (91).

ROS comprise a variety of diverse chemical species including superoxide anions (O_2•), hydroxyl radicals (OH•) and hydrogen peroxide (H_2O_2). Some of these species, such as superoxide or hydroxyl radicals, are extremely unstable, whereas others, like hydrogen peroxide are freely diffusible and relatively long-lived. These various radical species can either be generated exogenously or produced intracellularly from several different sources (90). Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria under the condition of ischemia. Mitochondria are the largest organelles in the cell and contain outer and inner membranes that define two compartments, the intermembrane space and the matrix. The outer membrane defines the smooth outer perimeter of the mitochondrion, and the inner membrane contains components of the electron transport chain (92). Mitochondria are the powerhouse of the cell and provide ATP through the oxidative phosphorylation process.

The proteins involved in oxidative phosphorylation include four multiple subunit enzyme complexes of the electron transport chain (complexes I-IV), ATP synthase (complex V), and the adenine nucleotide translocator (ANT) (Figure 1-1). Reduced nicotinamide adenine dinucleotide (NADH) is oxidized by complex I (NADH dehydrogenase), and succinate is oxidized by complex II (succinate dehydrogenase). The electrons collected at these sites are transferred to coenzyme Q (CoQ) to give reduced CoQ. Electrons from reduced CoQ are transferred to complex III (cytochrome bc1), then to cytochrome c (cyt c), followed by complex IV (cyt c oxidase, COX), and finally to oxygen (O_2) to give water. The energy generated by the electron flow is used to pump protons out of the matrix across the mitochondrial inner membrane. The resulting electrochemical gradient is used to reintroduce protons through the subunit F0 of complex V (ATP synthase) and to convert inorganic phosphate and ADP to ATP. The ATP generated in mitochondria, is then exchanged for cytosolic ADP by the ANT (93) (Figure 1-1).

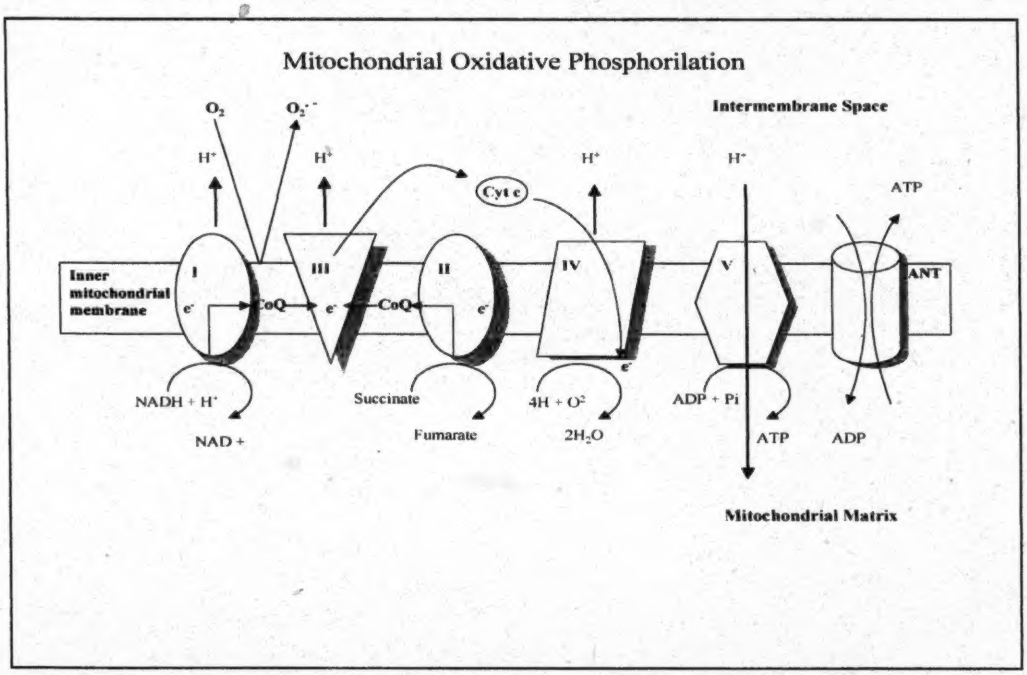

Figure 1-1. Mitochondrial Oxidative Phosphorylation (Electron transport chain). Cyt c: Cytochrome c; CoQ: Coenzyme Q; O2•-: Superoxide radical; H+: Hydrogen ion; Pi: Inorganic phosphate. (*As published by Jassem et. al. in Transplantation 2002; 73: 493-99*).

The production of mitochondrial superoxide radicals occurs primarily at two discrete points in the electron transport chain, namely at complex I (NADH dehydrogenase) and at complex III (ubiquinone-cytochrome c reductase). Under normal metabolic conditions, complex III is the main site of ROS production (94).

Approximately 90% of inhaled O_2 is consumed by mitochondria and reduced to water to produce energy. Under physiological conditions, each molecule of O_2 is reduced by four electrons (tetravalent reduction) at the level of complex IV, but 2% to 4% of the O_2 can capture single electrons that leak along the electron transport chain, particularly from oxidized CoQ (Univalent reduction) that form ROS. The formation of a free radical semiquinone anion species (•Q-) occurs as an intermediate in the regeneration of coenzyme Q. Once formed, •Q- can readily and non-enzymatically transfer electrons to molecular oxygen with the subsequent generation of a superoxide radical (93) (Figure 1-1).

After ischemia the electron transport chain complex may be damaged by a lack of substrate (O_2) resulting in a much greater leak of electrons. During reperfusion the re-introduction of O_2 reacts with leaking electrons to generate $O_2\bullet$, resulting in the "oxidative burst" (93).

Ischemic episodes of short duration increase the electronegativity of the electron transport chain complexes and leakage of electrons but may not alter mitochondrial antioxidant efficacy; thus, the ROS produced upon reperfusion are still inactivated by antioxidant mechanisms (95). Long periods of ischemia can significantly alter the electron transport complexes. All of the complexes show a reduction in their activity with structural damage to the subunits after 60 min of warm ischemia, but complexes I and III appear to be the most sensitive to ischemic injury (93). Extended warm ischemia also causes progressive reduction of the iron-sulfur proteins associated with complex I (NADII dehydrogenase) or complex II (succinate dehydrogenase) of the electron transport chain (Figure 1-1). This results in liberation of the ferrous iron (Fe^{2+}) that can be critical at reperfusion by reducing H_2O_2 and forming ROS (96) (Figure 1-2).

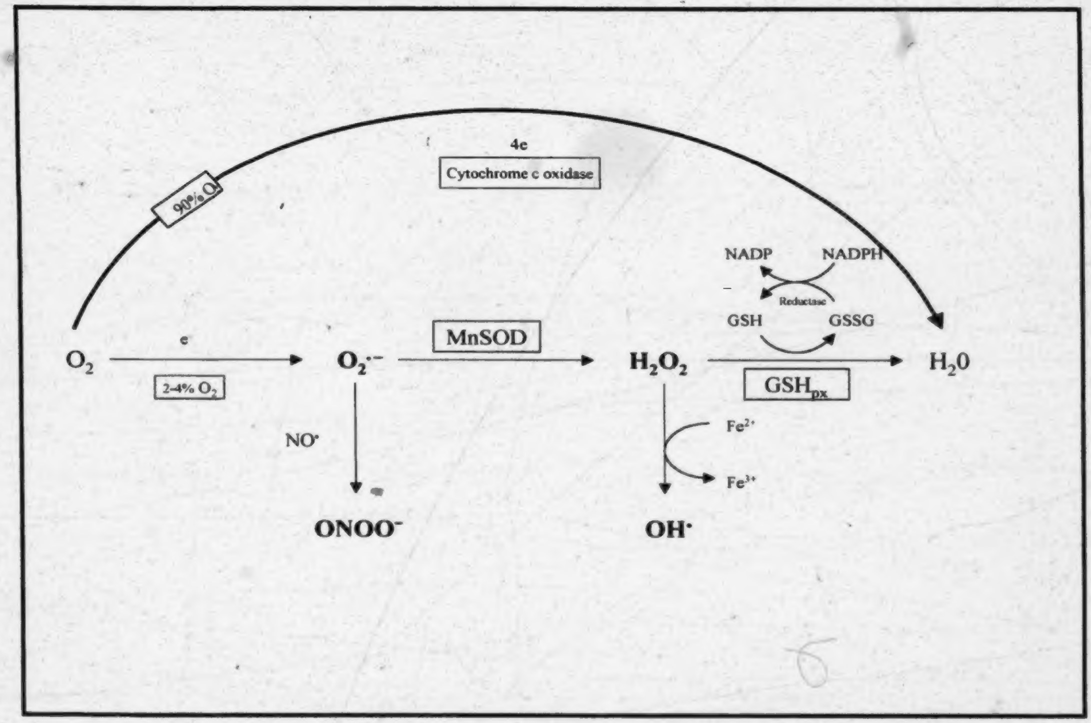

Figure 1-2. Generation of ROS and antioxidant activity in mitochondria after ischemia and reperfusion.

ROS: Reactive oxygen species; O_2: Oxygen; H_2O_2: Hydrogen peroxide; GSH_{px}: Glutathione peroxidase; $O_2\bullet$: Superoxide anion; MnSOD: Manganese superoxide dismutase; GSH: Reduced Glutathione; GSSG: Oxidized glutathione; NO•: Nitric oxide; $ONOO^-$: peroxynitrite anion; OH•: Hydroxyl radical; Fe^{2+}: Forrous iron; Fe^{3+}: Ferric iron. (*As published by Jassem et. al. in Transplantation 2002; 73: 493-99*).

Oxidative Modification Of Proteins, Lipids and DNA

The balance between ROS production and antioxidant defenses determines the degree of oxidative stress. Consequences of this stress include modification to cellular proteins, lipids and DNA. The most widely studied oxidative stress-induced modification to proteins is the formation of carbonyl derivatives (97). The dycarbonyls are very electrophilic compounds that react readily with the nitrogen of protein bound amino acids and of nucleic acids to give rise to advanced glycation endproducts (AGEs) (98). AGEs can also be formed by 4-hydroxy-alkenals (99), but most AGEs are formed by the interaction of such highly reactive dicarbonyls with protein. Modification of proteins by AGEs is associated with disease states such as diabetes, Alzheimer's disease and ageing (100). Hydrogen peroxide can react with transition metal ions to give rise to OH•, which is a powerful oxidant (Figure 1-2). Consequences of OH• formation are DNA damage and lipid peroxidation (101).

OH• can extract an electron from a polyunsaturated fatty acid, resulting in the formation of a carbon-centred lipid radical that can initiate a lipid peroxidation chain reaction by interacting with molecular oxygen and forming a lipid peroxyl radical. The lipid peroxyl radical in turn can extract an electron from another polyunsaturated fatty acid giving rise to a new carbon-centred lipid radical and a lipid hydroperoxide, thereby initiating a chain of lipid peroxidation (102). Formation of lipid peroxides affects membrane structure, resulting in altered membrane fluidity, increased permeability and decreased membrane ATPase activity (103). The Na+/K+ pump contains a labile lipid component that can be easily dissociated, including by lipid peroxidation (104). Lipid peroxidative products can also break down, forming strong oxidants including dicarbonyls such as malondialdehyde (MDA) and 4-hydroxyalkenals such as 4-hydroxynonenal. These are strong oxidants that can interfere with critical cellular functions such as mitochondrial respiration, as well as alter membrane protein configuration (102).

Oxidative damage can also affect DNA. One of the major means by which the OH• can cause cell damage is by causing changes in DNA leading to mutations (100). Perhaps because of its proximity to the main source of oxidant generation, or because of a limited DNA repair system, mitochondrial DNA is generally considered to be even more sensitive than nuclear DNA to oxidative damage. Increasing damage to mitochondrial DNA inevitably leads to compromised mitochondrial function and integrity. Damaged mitochondria are thought to release more ROS and set in motion a vicious cycle of increasing DNA damage leading to increased ROS production that in turn leads to more DNA damage (90).

Nitric Oxide

Other oxygen radicals with different sources of production and sites of action can also be deleterious to the cell. Nitric oxide (NO) is a reactive and unstable free radical gas that can cross cell membranes easily by diffusion without depending on any release or uptake mechanisms (105). It is a simple hydrophobic gaseous molecule that is highly diffusible and highly reactive and can diffuse into all directions passing through every cell membrane. NO is generated from a guanido nitrogen of L-arginine by at least three distinct isoforms of NO synthase (NOS) (106). NO is involved in several signaling pathways related to a diverse array of cell functions. A low level of NO synthesized by neuronal constitutive NOS for short periods of time acts as a neurotransmitter (105). Similarly NO produced by endothelial NOS plays a physiological role in regulation of vasodilatation and platelet aggregation (107). In contrast, high levels of NO produced by an inducible NOS (iNOS), mainly in macrophages and neutrophils, mediates cytotoxicity as the first line of host-defense against invading micro organisms or tumor cells (108). There is increasing interest in the effect of NO in insulin producing cells, especially β-cells. NO has been identified as a mediator of cytokine actions in islet β-cells (109). Cytokine interleukin-1β has been demonstrated to induce NO production in rat islet β-cells and a rat insulinoma cell line (RIN m5F), and NO production has been identified as a mechanism of IL-1-induced inhibition of mitochondrial enzyme activity, glucose oxidation and glucose-stimulated insulin release (110).

Oxidants In Cellular Signaling

Apart from the endogenous generation of ROS as a consequence of metabolic activities, many environmental stimuli including cytokines, ultraviolet radiation, chemotherapeutic agents, hyperthermia and even growth factors generate high levels of ROS that can perturb the normal redox balance and shift cells into a state of oxidative stress (90). When the stress is severe, survival is dependent on the ability of the cell to adapt to or resist the stress, and to repair/replace the damaged molecules. Alternatively, cells may respond to the insult by undergoing apoptosis. A number of stress response mechanisms have evolved to help the cell and organism adapt to acute stress and serve to coordinate the acute cellular stress response and ultimately determine the outcome (111).

Among the main stress signaling pathways and/or central mediators activated in response to oxidant injury include mitogen-activated protein kinase (MAPK) signaling cascades, the extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38, the phophoinositide 3 (PI (3)K)/Akt pathway, the NF-κB signaling system, p53 activation, and the heat shock response (Figure 1-3). Activation of these pathways is not unique to oxidative stress, as they are known to have central roles in regulating cellular responses to other stresses as well as regulating normal growth and metabolism. In general, the heat shock response, ERK, PI (3) K/Akt and NF-κB signaling pathways exert a pro-survival influence during oxidant injury, whereas activation of p53, JNK and p38 are more commonly linked to inflammation and apoptosis (112). Oxidants seem to activate the ERK and the PI (3) K/Akt pathways largely through stimulation of growth-factor receptors, mimicking the action of natural ligands. Many growth-factor receptors have been shown to undergo enhanced phosphorylation in response to direct treatment with oxidants, and agents or conditions that prevent receptor phosphorylation likewise inhibit the activation of ERK and Akt (113). The activation of growth factor receptor signaling pathways by oxidants is consistent with the demonstration that low concentrations of exogenous H_2O_2 can be mitogenic (114). Biochemical evidence indicates that under non-stressed conditions Glutathione S-transferase binds to JNK to inhibit its activation, but this interaction is also disrupted by oxidative stress (115). A common effect of the activation of these pathways is a change in pattern of gene expression mediated largely through modulation of the activities of transcription factors.

ERK activation exerts a pro-survival signal during oxidative stress; thus, reduced ERK activity in aged cells may have a negative impact on survival. It is also worth noting that activation of ERK in response to mitogenic stimulation is also reduced as a function of ageing and stress, emphasizing the intimate link between proliferative and oxidative stress-response pathways (116). Basal DNA binding activity of NF-κB has been shown to increase with age, reflecting an increased level of oxidative stress in aged cells and tissues (117).

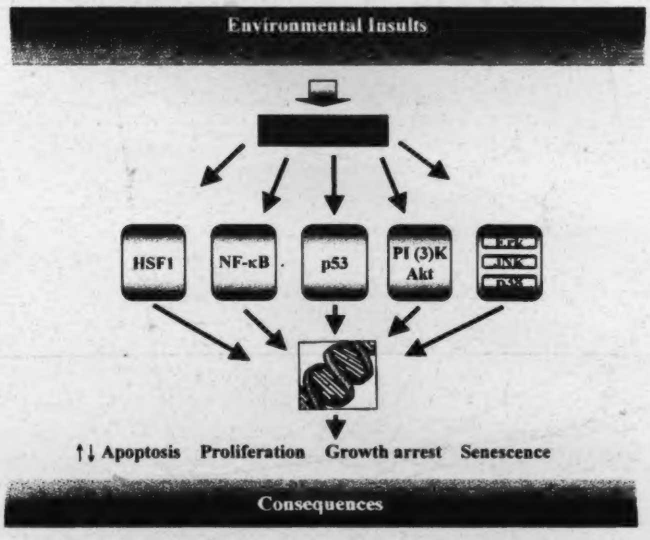

Figure 1-3. Major signaling pathways activated in response to oxidative stress and damage to DNA.

HSF1: Heat-shock transcription factor 1; NF-κB: Nuclear Factor Kappa B; PI (3)K/Akt: phophoinositide 3 pathway; ERK: Extracellular signal-regulated kinase; JNK: c-Jun amino-terminal kinase. *(As published by Finkel and Holbrook in Nature 2000; 408: 239-42).*

Antioxidant Mechanisms

The burden of ROS production is largely counteracted by an intricate antioxidant defense system that includes enzymatic and non-enzymatic elements. Enzymatic scanvengers include superoxide dismutase (SOD), catalase and glutathione peroxidase (GSHpx). SOD speeds the conversion of $O_2•$ to H_2O_2, whereas catalase and GSHpx convert H_2O_2 to water (Figure 1-2). Glutathione peroxidase converts H_2O_2 into water using reduced glutathione (GSH) as a substrate to produce oxidized glutathione (GSSG) (Figure1-2). The ratio between GSH/GSSG is an important marker of the redox state of the cell. Mitochondrial GSH is separate from the cytosolic pool and is present mainly in a reduced form at high concentrations in the mitochondrial matrix (93).

Glutathione peroxidase, remain relatively stable during ischemia, although GSH levels may decrease during both warm and cold ischemia (93). Mitochondrial Superoxide Dismutase MnSOD seems to be more susceptible to ischemia than cytosolic copper and zinc superoxide dismutase (Cu, ZnSOD). After 60 min of global ischemia, MnSOD activity is significantly depressed (118).

A variety of other non-enzymatic, low molecular mass molecules are important in scavenging ROS. These include ascorbate, pyruvate, flavonoids, and carotenoids, but the most important is GSH, which is present in millimolar concentrations within cells (111). GSH is a tripeptide (γ-glutamyl-cysteinyl-glycine). Since cysteine contains a sulfhydryl residue, and it is easily oxidized, GSH behaves as a very efficient sink of ROS. In a typical redox reaction, a ROS is reduced (and inactivated) through the generation of a disulfur bond between two GSH molecules, yielding the oxidized GSH pair. Once the ROS has been inactivated, two reduced GSH molecules can be recovered through the enzyme reaction catalized by GSH-reductase (119). Glutathione is an important water-phase antioxidant and essential cofactor for antioxidant enzymes;

it provides protection also for the mitochondria against endogenous oxygen radicals. It has a high electron-donating capacity and high intracellular concentration, which gives GSH a great reducing power. GSH depletion may be the ultimate factor determining vulnerability to oxidant attack.

Glutamine, a precursor of GSH is able to enter the cell more readily than glutathione (120). Glutamine, is the most abundant amino acid in the blood of mammals and it has been described as the main nitrogen vehicle among the tissues of the organism (121). Several transport systems, in both plasma membrane and inner mitochondrial membrane warrant the efficient uptake of glutamine into mitochondria, where it is metabolized (122). The first step in the metabolism of glutamine is its deamination by phosphate-activated glutaminase, yielding glutamate and ammonia (123). Glutamate is transported back to the cytosol and there it can be used in the synthesis of GSH. Glutamine supports the intracellular pool of glutamate, avoiding its depletion and the depletion of GSH. Glutamine can be a means of providing two of the three precursors necessary for GSH synthesis; that is, glutamine itself can be considered a precursor molecule to GSH (119).

Apoptosis and Necrotic cell death

Cell death can follow two distinct pathways, apoptosis or necrosis. Although apoptosis and necrosis have long been viewed as opposed, it is now generally assumed that both forms of cell death constitute two extremes of a continuum (124). An example is that the same toxin can induce apoptosis or necrosis at a low or high dose, respectively (125). The equilibrium between apoptosis and necrosis can be influenced by manipulation of ATP levels and caspase activation, thus maintenance of high ATP favors apoptosis over necrosis (126), whereas inhibition of caspase activation may transform apoptosis into necrosis (127).

Necrosis appears to be the result of acute cellular dysfunction in response to severe stress conditions or after exposure to toxic agents, and is a relatively passive process associated with rapid cellular ATP depletion (128). In contrast to apoptosis, necrosis does not involve any regular DNA and protein degradation pattern and is accompanied by swelling of the entire cytoplasm and of the mitochondrial matrix, which occur shortly before the cell membrane ruptures (129). Morphologically, necrosis is characterized by a dramatic increase in cell volume and rupture of the plasma membrane, with spilling of the cellular contents into the intercellular milieu. This release of the dying cell contents into the extracellular space can cause further tissue damage by affecting neighboring cells or by attracting proinflammatory cells to the lesion (130).

Apoptosis is a form of cell death that occurs during several pathological situations in multicellular organisms and constitutes a common mechanism of cell replacement, tissue remodelling, and removal of damaged cells (131). In contrast to necrosis, apoptosis involves the regulated action of catabolic enzymes (proteases and nucleases) within the limits of a near-to-intact plasma membrane (124). Apoptosis is a complex process characterized by cell shrinkage, chromatin condensation, internucleosomal DNA fragmentation, and the formation of "apoptotic bodies" (128).

Several protease families are implicated in apoptosis, the most prominent being caspases (132). Caspases are cysteine-containing, aspartic acid-specific proteases which exist as zymogens in the soluble cytoplasm, mitochondrial intermembrane space, and nuclear matrix of virtually all cells (133). Caspase activation and proteolytic cleavage of specific target proteins with structural, regulatory or housekeeping functions represents an integral step in the pathway leading to the apoptotic death of cells (134). Caspases can be placed into three broad categories: those such as caspase 1 that are primarily involved in inflammatory responses; and two groups of caspases that are involved in apoptosis: initiator capases such as caspase 8 and effector caspases such as caspase 3 (135).

Apoptosis and Necrosis related to Oxidative Stress

For many years, direct treatment of cells with oxidants like hydrogen peroxide or redox-active quinones was thought to exclusively cause necrosis, but more recent studies have shown that lower doses of these agents can trigger apoptosis (136). Intracellular ROS generation has been shown to constitute a conserved apoptotic event and a critical determinant of toxicity associated with exposure to ionizing radiation and chemotherapeutic drugs with oxidative properties (137). Moreover, depletion of GSH pools has also been suggested to be part of the cell death effector machinery and accompanies ROS production during apoptosis in relevant systems (138). Some groups have shown drops in intracellular GSH levels and concomitant increases in ROS during Fas-induced apoptosis (139, 140). Redistribution of cellular GSH may be critical during apoptosis; although GSH is synthesized in cytosol, it is transported into organelles, including the mitochondria and the nucleus, where it can be used as a cofactor in glutathione peroxidase and S-transferase-mediated reactions (128).

Endogenous mediators, such as ROS, which interfere with the death program, can decide the mode of cell death. Apoptotic cell death can be switched to necrosis during oxidative stress by two possible mechanisms. First, inactivation of caspases due to oxidation of their active site thiol group by oxidants (141, 142). The second mechanism is a drop in cellular levels of ATP due to the failure of mitochondrial energy production by oxidants (143). Overall, the activity of caspases is optimal under reducing environments, so any deviation from such reducing conditions (oxidation) within an injured cell could be detrimental and inactivate them. The consequences of mitochondrial dysfunction (collapse of the mitochondrial inner transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix calcium and GSH, and release of soluble intermembrane proteins) entails a bioenergetic catastrophe resulting in the disruption of plasma membrane integrity (necrosis) and /or the activation of specific apoptogenic proteases (caspases) by mitochondrial proteins that leak into the cyotosol (cytochrome c, apoptosis-inducing factor) with secondary endonuclease activation (apoptosis) (124).

Oxidative Stress and Apoptosis in Insulin Secreting Cells and Diabetes.

Pancreatic islets of Langerhans are extremely sensitive to oxidative stress because the activity of the GSH peroxidase as well as the GSH/GSSG ratio is low compared to other tissues (144). A decrease of the GSH/GSSG ratio causes inhibition of calcium uptake and insulin secretion. Therefore, oxidative stress to β-cells may be of relevance during the process of β-cell destruction in the development of type 1 diabetes (145). Insulinoma cells, an insulin secreting cell line, were found to develop apoptosis following oxidative stress as a consequence of the lysosomal burst that in turn resulted from intralysosomal iron-catalyzed oxidative reactions (145). In work done by Olejnicka *et al* (146), it turned out that a limited lysosomal rupture results in apoptosis, whereas a more pronounced lysosomal rupture leads to necrotic cell death. They found that the sensitivity of pancreatic islets to damage by oxidative stress induces lysosomal burst and consequent apoptosis or necrosis depending on the magnitude of the lysosomal burst.

Based on the vascular structure of the pancreas, islets seem to be the first-pass tissue of blood-borne toxic agents into the pancreas (147). In a study done by Ulrich *et al* (147) it was suggested that the predominant distribution of one of the isotypes of GSH-S-transferase enzyme in islets may be explained by the anatomic blood supply of the pancreas. Generally, the arterial blood passes through the islets first before nourishing the exocrine pancreas. Acinar cells, in contrast, do not have their own direct blood supply. Consequently, islet cells appear to take on the burden of clearing toxic substances that reach the pancreas. An increase of detoxifying enzymes in islet cells compared to the rest of the pancreatic tissue could indicate overexposure of the pancreas to toxic agents. Nevertheless, the presence of antioxidant enzymes in islets has been found lower than in various other tissues (148), rendering them more vulnerable to oxidative stress than any other tissue.

Part 4) Artificial O_2 Carriers

Artificial O_2 carriers are synthetic solutions capable of binding, transporting and unloading O_2 within the body. They have originally been developed as alternatives to blood transfusion, or to improve tissue oxygenation of organs with marginal O_2 supply. O_2 carriers can be classified into two broad categories: 1) Perfluorocarbons (PFCs) and 2) Hemoglobin-based O_2 carriers (HBOCs).

Perfluorocarbons

Perfluorocarbons were originally produced with the intention to serve as a blood substitute during World War II. The first reports about its synthesis and chemistry appeared in "Nature" in 1951 (149), and a description of its potential uses appeared later in 1966, in a publication of the journal "Science", in a dramatic report that described rodents totally immersed and breathing in this fluid (150). PFCs are compounds capable of dissolving O_2 under conditions that make it biocompatible even for administration into the intravascular space (151). Intravascular O_2 carriage is directly proportional to both the partial pressure of O_2 (PO_2) in the blood and the concentration of PFCs in the blood (152) .

Apart from their original use as blood substitutes, PFCs have been used as organ preserving solutions (153), having great success described for pancreas and islet preservation using the TLM in combination with UW Solution (154). The storage of organs with PFCs has been shown to increase ATP levels, which is associated with decreased levels of MDA and oxidative injury (155). Major islet transplantation programs in North America have already included PFCs and the TLM into their protocols (156-159). A large drawback with PFCs, though, is their viscosity; to date, more emulsified solutions have been difficult to obtain.

Properties of PFC:

- Chemically inert
- Colorless
- Odorless
- Contain high gas solubility
- High density
- Low Viscosity
- Low surface tension
- Radio-opacity
- High compressibility
- Immiscible in aqueous systems

Hemoglobin-based O_2 Carriers

Hemoglobin-based solutions use free (extra-erythrocytic) hemoglobin (Hb) derived either from red cells or biosynthesized de novo.

Hb in the red blood cells exists as a tetramer composed of two alpha and two beta globin chains ($\alpha_2\beta_2$). Each chain holds an iron-containing porphyrin ring and each iron atom can bind reversibly with the O_2 molecule. HBOC solutions can be prepared by releasing Hb from bovine or time-expired human erythrocytes, or biosynthesized using recombinant techniques (160). Initial preparations used intravenously were related to a number of toxic effects including vasopressor action, activation of complement and coagulation pathways, nephrotoxicity and histamine release among others (161), but by the 1970's, new purification techniques allowed the production of a "highly purified" Hb that resolved many of the early adverse effects. However, this new generation of purified Hb was still associated with some problems that limited their use like 1) higher saturation and avidity for O_2 resulting in lower release, 2) unstable free Hb, which quickly dissociates into $\alpha\beta$ dimmers which are excreted in the urine, with short intravascular half-life (1-2 hours) and causing nephrotoxicity, 3) the ferric group in free Hb is an avid scavenger of NO, resulting in an increase in systemic and pulmonary pressures, 4) O_2 dissociating from free Hb can uncouple an electron, creating methemoglobin and a superoxide radical, which causes oxidative stress. HBOCs are easily oxidized to methemoglobin and must be stored anaerobically.

Cross-linking, conjugation or microencapsulation of extracted Hb, or genetic engineering, can overcome many of these problems (162). Human-derived HBOCs, produced by lysis of erythrocytes obtained from outdated banked blood, have a P_{50} (the partial pressure of oxygen which results in 50% oxygen saturation of Hb) reduced from 27 mmHg to 12-13 mmHg. Hb cross-linkage, pyridoxylation or carboxymethylation of the terminal Hb residues can reduce this high O_2 affinity. However, human-derived HBOC solutions are produced from the limited available stores of donated blood. An alternative to this approach is the use of animal-derived Hb, which has a P_{50} of around 28-30 mmHg (163), releasing O_2 more readily to the tissues. Prolonging the intravascular retention and reduction of colloid osmotic pressure can be achieved by a number of processes. Colloid osmotic pressure is proportional to particle number rather that size. Increasing particle size by polymerization of Hb increases the number of O_2 binding sites relative to the osmotic pressure. Polymerization of Hb molecules by glutaraldehyde or raffinose results in the formation of stable tetramers and of tetramer polymers. Polymerization, however, can reduce O_2 binding cooperativity (161).

Encapsulation of Hb is another alternative recently introduced to produce pseudo-erythrocytes in order to prolong intravascular retention (164). Recombinant techniques allow modifications to the globin chains expressed leading to a reduction in O_2 affinity and maintenance of the tetrameric configuration after the infusion (164).

A number of HBOC have completed safety studies, and already phase III studies to test efficacy are well underway (165).

Properties of HBOCs:

- Unlimited supply
- Prolonged storage
- High chemical Purity
- No blood matching required
- Enhanced solubility
- Physiological O_2 release

The relationship between the PO_2 and the oxyhemoglobin saturation follows a well-known S-shaped curve. This curve can be shifted to the right or left depending on the concentration of the allosteric modifier, 2,3, diphosphoglycerate (2,3 DPG), in the red cells; less 2,3 DPG shifts the curve to the left. The curve is also shifted and the P_{50} for half-saturation value increased by ambient conditions such as increase in body temperature, decrease in pH or increase in carbon dioxide tension (PCO_2). Decreases in temperature and PCO_2 or increases in pH have an opposite effect and the oxyhemoglobin dissociation curve shifts to the left (Figure 1-4).

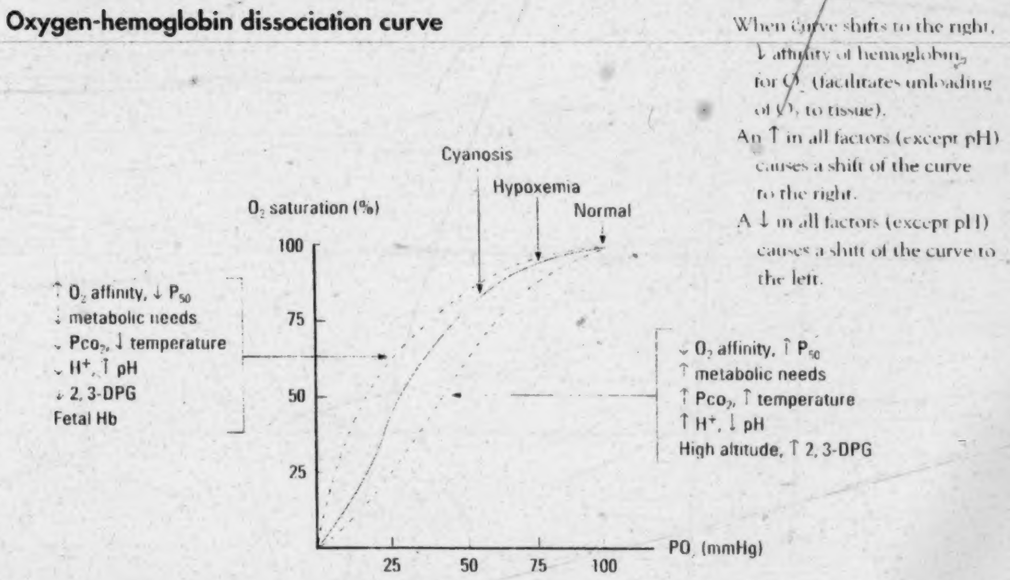

Figure 1-4. Oxygen-hemoglobin dissociation curve and factors affecting it. (*Modified from Harper's Illustrated Biochemistry, 2003*).

The amount of O_2 that can be bound by one gram of Hb is approximately 1.34 ml (152). O_2 content of the plasma, in contrast to that of hemoglobin, is directly related to the PO_2. Solubility of O_2 in plasma is approximately 0.3 ml/dL per 100 mmHg PO_2. In the case of PFC, O_2 transportation is performed in the same way as plasma, ie., as a direct function of their solubility coefficient and the PO_2. The differences between Hb and PFC are depicted in (Figure 1-5), which demonstrates the relationship between O_2 content and PO_2 in blood with a normal Hb of about 14 g/dL, fully saturated and containing about 20 ml/dL of O_2. At a normal ambient PO_2 at sea level of about 150 mmHg, the patient with normal pulmonary function will be able to achieve a P_{50} of approximately 100 mmHg, sufficient to ensure about 98% oxyhemoglobin saturation. As PO_2 is decreased to about 40 mmHg, about the level of the mixed venous O_2 tension, approximately 20 to 25% of the O_2 bound to Hb (4-5 ml/dL) will be released to the tissues. At this PO_2, PFC will release 60-90% of its dissolved O_2, which is approximately 4-5 ml/dl, but as PO_2

increases over 100 mmHg, the amount of O_2 released by Hb remains constant, whereas O_2 released from PFC will gradually increase in a straight line (152).

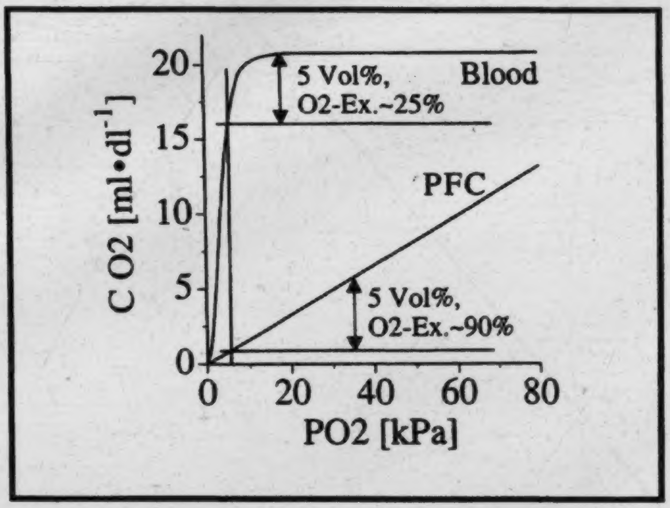

Figure 1-5. O_2 binding and delivery characteristics of blood and PFC emulsion.
C O_2: Total O_2 content; O_2 Ex.: Extraction of Oxygen; PO_2: Partial O_2 pressure; Vol %: ml/dl of Oxygen. (*Faithfull N.S. Oxygen Transport to Tissue XXIV, Chapter 26. Kluwer Academic/Plenum Publishers, 2003*).

The appropriate HBOCs O_2-transport parameters have not yet been completely determined. The O_2 affinity is an important factor in determining O_2 delivery to tissues (166). HBOCs with an O_2 affinity similar to or lower than that of red blood cells should facilitate O_2 unloading to the tissues. Experimental studies have clearly demonstrated that HBOCs can improve tissue oxygenation (167-170). The fact that they bind and release O_2 in a similar way to Hb contained in healthy red blood cells makes HBOCs an attractive compound to use in organ preservation, where the amount of O_2 delivered can be critical to an already ischemic tissue.

Conclusions

The deleterious effects of ischemia and oxidative stress in pancreatic islets are clearly major factors that influence the success of islet isolation and transplantation.

After the advent of the Edmonton Protocol, islet transplantation seems an encouraging therapy for patients with diabetes, especially if we take into account the rate of complications for whole pancreas transplantation. However, it should be balanced with a reduced long-term endocrine function and an increased number of donors required for achieving insulin-independence. In order to improve the efficiency of islet transplantation, the availability of islets has to be increased. Tissue damage as a result of ischemia is an important factor in the outcome of the isolation, which can potentially occur at organ procurement, preservation, and the proper isolation procedure.

The production of ROS and oxidative stress are recognized as an important source of islet cell injury and come mostly as result of ischemia on mitochondrial structures. They are also involved in the generation of intracellular signals triggered during the isolation, leading to cell death in insulin producing cells either through apoptosis or necrosis. The use of antioxidants is effective in counter acting the production and effects of ROS in pancreatic tissue, protecting islets from oxidative injury during isolation. Although antioxidant enzymes are lower in pancreatic islets compared to other tissues, they seem to maintain a stable function under ischemic conditions, experiencing a decrease in their substrate (GSH). This suggests that maintaining the stores of GSH would improve antioxidant mechanisms in islets promoting recovery and protecting them against oxidative stress and cellular damage.

Availability of O_2 may be deleterious to the cell once mitochondrial damage is irreversible. When the damage is still reversible, though, mitochondria may use the oxygen for the replenishment of energy stores (ATP), reducing the injury produced by ischemia.

The purpose of this project is to improve islet isolation and transplantation outcomes by intraductal distention of the pancreas with different solutions after a period of ischemia. One goal is directed to the restoration of the islet cell's GSH levels by providing L-Glutamine, one if its precursors. We expect that the islets restore their defense mechanisms against oxidative injury by increasing the levels of GSH, therefore, improving islet yield, viability and function after isolation and transplantation. The second goal is directed to provide physiological levels of O_2 after a period of warm ischemia. In this context, we expect that the use of O_2 carriers would increase tissue oxygenation and provide substrate for the protection of energy production in the cell. Therefore, recovering and maintaining the integrity of mitochondrial membranes and protecting islets against further damage.

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Chapter 2

Methodology

General Experimental Procedures

Islet Isolations

Human Islet Isolation

Human pancreata refused by other centers for whole pancreas or islet transplantation were obtained from organ procurement organizations (Gift of Hope, Illinois; Gift of Life, Michigan; and Indiana). Pancreata were obtained from brain dead donors using standard organ procurement techniques by the liver team responsible and sent to the Cell Isolation Laboratory at the University of Illinois at Chicago (UIC) where they were processed as described extensively (1, 2). Briefly, the pancreas is cleaned from the surrounding fat and fibrotic tissue and weighed. The pancreatic duct is canulated and perfused with a cold Liberase solution, 2 mg/ml, in 250 ml of Hanks' solution (Liberase-HI; Roche, Indianapolis, IN). The distended pancreas is placed in the digestion chamber (capacity 500 ml), which has been pre-warmed to 29°C. A double stainless screen (500 μm and 1000 μm meshes) is placed between the lower and the upper portion of the chamber to retain undigested tissue. The chamber is then connected to the tubing (circulating system activated by a peristaltic pump) and warmed up to 37°C. The chamber, containing marbles, is shaken continuously and gently agitated during the digestion. Samples of the preparation are taken regularly from a tap. The decision to stop the enzymatic digestion depends on the detection of free islets, the amount of digested tissue and the size of exocrine tissue in the dithizone stained samples. The digestion is stopped by dilution and cooling, and the digested pancreatic tissue is collected, then centrifuged, and the final islet preparation is resuspended.

Samples of the preparation are taken for pre-purification islet counting. For islet counting only intact, dithizone stained islets are taken into consideration and the equivalent islet number (EIN) is calculated by normalizing the islets to a standard islet of 150 µm diameter as previously described in detail (3). Sterile conditions are maintained throughout the whole procedure. After incubation in UW-solution (Viaspan), islets for allotransplantation are purified by a continuous Bio-Ficoll gradient (Mediatech) on a cell separator (Cobe 2991, Cobe, Lakewood, CO). Islets are cultured in supplemented CMRL and 5% CO_2 at 24° or 37° C in non-adherent culture flasks until transplanted.

Purified Collagenase Enzyme (Liberase) with L-Glutamine for Human pancreata

Pancreas digestion was performed using Liberase-HI; (Roche, Indianapolis, IN). The enzyme was dissolved with cold Hanks balanced salt solution (HBSS) for all control groups. The enzyme for Glutamine treatment groups was dissolved in HBSS containing a final concentration of 5mM L-Glutamine (Combine 12.5ml of 200mM L-Glutamine (Invitrogen) with 488ml of Perfusion Solution (Mediatech)). Prior to digestion, pancreata were manually perfused (syringe loaded) through the main pancreatic duct with either the standard HBSS (control) or with HBSS + 5mM L-Glutamine (treatment), and left at least 15 min before its transfer to the digestion chamber.

Rat Islet Isolations and Surgical Procedures

Male Lewis rats (Harlan Industries, Indianapolis, IN), weighing between 175-200g were used as pancreas donors for islets. All animals were housed at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC). Animals were anesthetized by inhalation with isoflurane using an isoflurane vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ. U.S.). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Rat islet isolation was performed following the standard method previously described (4), modified by using the warm ischemia model that we have developed and described (5). Briefly, after making sure that the animal was properly anesthetized through a “foot pinch” technique, a laparotomy incision was performed followed by incision into the thoracic cavity and section of the heart in order to euthanise the animal by exsanguination. The abdominal cavity was closed, covered with gauze and left for 30 min before pancreas perfusion. After this period, 10ml of either “control” or “treatment” solutions were injected via the bile duct and into the main pancreatic duct for distention of the pancreas. After excision, each pancreas was placed in a 50 ml conical tube with 7.5 ml of its respective perfusion solution followed by incubation in a 37°C water bath (digestion phase) for 18 minutes. After this step, each pancreas was gently shaken in the tubes, washed with cold HBSS and transferred into a 500 ml beaker through a 500 μm filter for further washing. After a thorough set of 3 washes and centrifugation at 344 g for 1 min each, islets were purified from the exocrine tissue by discontinuous Ficoll density gradients (Mediatech Inc. Herndon, VA). Islets were centrifuged for 15 min at 640 g and then handpicked from the middle layer of ficoll, followed by thorough washing (3 times) and subsequent plating in RPMI containing 10% fetal calf serum (FBS), 10% Penicillin/Streptomycin (Invitrogen) with no glutamine, for 24 hours culture at 37°C.

Preparation of Collagenase Enzyme (Type XI) with L-Glutamine for Rat pancreata

Rat pancreas digestion was performed using collagenase type XI, (Sigma). The enzyme was dissolved with cold Hanks balanced salt solution (HBSS) for control groups. The enzyme for Glutamine treatment groups was dissolved in HBSS containing a final concentration of 5mM L-Glutamine (Combine 25ml of 200mM L-Glutamine (Invitrogen) with 975ml of HBSS (Mediatech)). Following euthanasia and after 30min warm ischemia to the pancreas, 10 ml of the enzyme (1mg/ml) were manually (syringe) loaded through the bile duct and main pancreatic duct, with either the standard HBSS (control) or with HBSS + 5mM L-Glutamine (treatment), and transfered to the water bath (37°C) for digestion.

Preparation of Collagenase Enzyme (Type XI) with Human Polymerized Hemoglobin (PolyHeme) for Rat pancreata

Preparation of the collagenase enzyme with PolyHeme for rats followed the same principle as with the Glutamine. Polymerized human hemoglobin was manufactured and kindly donated by Northfield Industries, Evanston, Illinois. PolyHeme containing Hb 10 g/dl was custom prepared for our isolation model by using RPMI-1640 reconstituted from powder (Mediatech Inc. Herndon, VA) in order to provide a medium suitable for dissolving the collagenase enzyme type XI (Sigma) at a concentration of 1mg/ml. Previous experiments were performed at Northfield laboratories to determine that collagenase activity does not interfere with PolyHeme through the formation of toxic dimmers, methemoglobin (MetHb) or carboxihemoglobin (COHb) at toxic levels. 250 ml of this solution were packed sterile in 500 ml bags to allow for the introduction of 250 ml of O_2. The bags, with a shelf life of 12 months were stored at 4°C for no more than 9 months. Manual syringe loading of the pancreata was performed following 30min of warm ischemia after euthanasia of the animal. The pancreata were then transferred to a water bath at 37°C for digestion.

Assessment of Islet Isolation Outcomes

In Vitro Assessment

Islet Yield: The outcome of the islet isolation was assessed through the quantification of islet mass by dithizone staining and counts expressed in equivalent islet numbers (EIN). Two independent investigators assessed the quantity and purity of the preparations in accordance to the criteria established at the 1989 International Workshop on Islet Assessment (3).

Islets from rat pancreata followed a similar assessment, stained with dithizone, but counted by number of single islets instead of Islet Equivalents.

Viability: Islet viability was assessed by fluorescent staining with Syto-Green/Ethidium Bromide (EB) (6), and the percentage of dead and live cells was estimated in both, control and treatment groups as previously described (5). Syto Green was prepared by adding 20μl of Syto-Green (Molecular Probes) to 980μl of Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen) without calcium and magnesium chloride. EB wass prepared by adding 10μl of Ethidium Bromide (Sigma) to 990μl of DPBS. A sterile sample of at least 100 islets was washed twice with DPBS and transferred into a counting dish (approximately 3ml) were 200μl of Syto-Green were added, followed 3 minutes later by 100μl of EB and counted. Double fluorescence staining was performed to assess the amount of live (green) versus dead (red) islet cells in a representative sample, where a minimum of 100 islets were counted per sample. Islets that were stained over more than 25% of its surface were considered dead.

Islet viability was also assessed by staining with trypan blue dye (Sigma). A minimum of 100 islets were counted in 3ml of media with 100μl of trypan blue. Islets stained over more than 25% of its surface were considered dead. Live versus dead islets were assessed in a representative sample, where a minimum of 50 islets were counted per sample

Function: In vitro islet function was performed by static glucose incubation and expressed in terms of stimulation index (SI) by calculating insulin secretion of islets challenged with Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.35) containing 10 mM HEPES and 0.5% BSA (Sigma) with high glucose concentration (16.7mM) and dividing it by insulin secretion under low glucose conditions (1.6mM) (7). Briefly, groups of 5 handpicked islets identified under a stereoscopic microscope were placed in five different wells of a 12 well-plate; then incubated with 1ml of KRBB-low glucose concentration (1.6 mM glucose final concentration) for 30 min, allowing them to stabilize insulin secretion, then the supernatant was collected and discarded. Islets were then incubated for 1 hour in low glucose KRBB (1.6 mM glucose final concentration) at 37° C and 5% CO_2, and supernatants were collected under a microscope taking care to avoid removing any islets from the well. The same step was repeated by addition of KRBB-high glucose solution (16.7 mM glucose final concentration) and incubation for 90 min. Supernatants were collected and frozen at -20°C for later measurement using an ELISA kit for human insulin (Mercodia; Uppsala, Sweden).

Measurements of intracellular Ca^{2+} for in vitro function: Dual-wavelength excitation fluorescent microscopy was used to measure intracellular Ca^{++} levels as described (8). Islets (25 to 30 per isolation) were incubated in 6 well-plates with a 96cm cover slip for 48 hours in standard culture media (RPMI-1640) immediately after isolation. Islets were loaded with a fluorescent stain (Fura-2) by a 25-min incubation at 37°C in Krebs solution with 2 mM glucose (KRB2) containing 5 mM Fura-2 (Molecular Probes Inc., Eugene, OR), then placed into a temperature-controlled perfusion chamber (Medical Systems Inc.) mounted on an inverted fluorescence microscope (TE-2000U, Nikon, Inc.) and perifused by a continuous flow (rate 2.5 ml/min) of 5% CO2-bubbled KRB2 buffer at 37°C (pH 7.4). KRB containing different glucose concentrations (5, 8, 14 mM) was administered to the islets after rinsing in KRB2 for 10-15 min in between each concentration. Single islets were visualized with a 40x oil-immersion quartz objective and multiple islets simultaneously imaged with 10x-20x objectives. Fura-2 dual-wavelength excitation at 340 and 380 nm, and detection of fluorescence emission at 510 nm was performed. Analysis software was used (Universal Imaging Corporation); images were collected with a high-speed, high-resolution imaging device (Roper Cascade CCD). Estimation of the level of Ca^{2+} was accomplished by calibration through islets without glucose stimulation (9).

Addition of Tolbutamide: Tolbutamine, an inhibitor of K^+ channels (100 mM concentration) was added after 5 minutes to the perifusion medium containing islets in Krebs (2 mM glucose). The measurement of intracellular Ca^{2+} in islets was performed until 50 minutes after addition of Tolbutaminde at basal conditions without glucose stimulation. Calcium measurement and imaging was performed as described above.

β-Cell metabolic assessment through mitochondrial membrane potential: The fluorescent dye Rhodamine 123 (Rh123) is a probe of the transmembrane potential and is accumulated within the inner mitochondrial membrane (Rh123 is a lipophilic cation that integrates selectively into the negatively-charged mitochondrial membranes). In cells pre-loaded with Rh123, when membrane potential increases (hyper-polarization) as seen after glucose stimulation, more Rh123 is concentrated into the mitochondrial membrane, leading to aggregation of dye molecules and decrease (quenching) of the fluorescence signal. Rh123 was used as previously described (34). Briefly, islets were incubated in Krebs solution with glucose (2mM), supplemented with 10 μg/mL Rh123 for 20 min at 37°C, then placed into a temperature-controlled perfusion chamber (Medical Systems Inc, Paola, KS) mounted on an inverted epifluorescence microscope (TE-2000U, Nikon Inc, Melville, NY.) The islets were perifused by a continuous flow (rate 2.5 ml/min) of 5% CO_2-bubbled Krebs buffer at 37°C (pH 7.4). Islets were then stimulated with 14 mM glucose and the changes in fluorescence were measured for 15 min after stimulation. Rh123 fluorescence was excited at 540 nm and emission measured at 590 nm. Images were collected with a charged coupled device camera (Roper Cascade CCD). Data was normalized to the average fluorescence intensity recorded during a five-minute period prior to glucose stimulation. All experiments were performed at 37°C.

Confocal Microscopy (Mitochondrial Morphology): Islets (15-20) were incubated for 48 hours in an 8-chamber coverglass (Nalge Nunc International, NY) in standard media (RPMI-1640) to allow them to adhere to the bottom surface. Islets were then incubated with Rhodamine 123 at 2.5 μM in Krebs buffer (2 mM glucose) for 15 minutes and visualized using a Carl Zeiss LSM 510 confocal microscopy with 60 X water immersion objective. The 488 nm beams from an argon-krypton laser used for excitation and the emission from Rh 123 were detected through an LP 505 filter.

Oxidative Stress: GSH levels were measured using the monochlorobimane (mcbm) method (10) in islets at 12 hours post-isolation as previously described (5). Briefly, (500 islets from rats) and (1,000 EIN for human pancreata) were cultured in one well of a 12 well-plate in 5 ml CMRL culture medium and 10 μL of mcbm (50mM) (Molecular Probes; Eugene, OR, U.S.) and were incubated for 30 min at 37°C. Islets were collected, washed with phosphate buffered saline (PBS) at a pH of 7.5, resuspended in 500 μL of 50mM TRIS buffer containing 1mM EDTA (1mL), then sonicated. After centrifugation the supernatant was read in a fluorescence plate reader (GENios, Tecan US Inc. (Durham, NC)) with excitation set at 380 nm and emission set at 470 nm.

Oxidative injury: Lipid peroxidation was used as a marker of oxidative injury, determined by measurement of malondialdehyde (MDA). MDA is a product of lipid peroxidation. MDA levels were assessed by the Thiobarbituric acid method (TBARS) (11). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67 % TBA, 10 % phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all Sigma). (500 islets from rats) and (1,000 EIN from human pancreata) were sonicated in 700μl PBS into a cell lysate. After centrifugation at 20142 g, 500 μl of the supernatant were extracted and mixed with 875 μl of the reaction mixture, then boiled at 95-98°C for 1 hour. After this process, samples were cooled down and mixed with 750 μl of N-butanol in order to extract the MDA and avoid interference of other compounds. After a short spin, 100 μl of supernatant were extracted and read in duplicates on a 96 well plate with a fluorometer

(GENios, Tecan US Inc. (Durham, NC)) set at Excitation: 530/25, Emission: 575/15. Samples were read against MDA standards (Sigma) prepared at different concentrations (2, 4, 8 mM).

Apoptosis for human pancreata: was assessed through Immunohistochemical Analysis (Detection of free 3-OH strand breaks resulting from DNA degradation by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). In order to assess the influence of treatments on islet cell apoptosis, islets from different human pancreata were fixed, sections performed and stained for apoptosis. (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon International, USA). 5 sections from each pancreas per day (1 and 3) were stained and counted; a minumum of 30 islets per section were randomly counted by field. The comparison of apoptotic vs. live cells was performed for the experimental groups.

Apoptosis for rat pancreata: The level of apoptotic cell death was measured using a living cell fluorescein active caspase-3 staining kit (Biovision, California). Briefly, an aliquot of 1,200 islets per group was counted and divided into 4 Eppendorf tubes with 300 μl of media each (RPMI 1640 with 10% FBS and 10% Pen/Strep). Following, 1 μl of FITC-DEVD-FMK (fluorescent dye for Caspase-3) was added into 2 of the tubes of each group (the other 2 of each group were left without the probe as controls for the assay) and incubated for 1 hour at 37°C and 5% CO_2. Cells were centrifuged at 344 g for 1 min and supernatant removed. Cells were then resuspended with the wash buffer from the kit, repeating this step twice, followed by resuspension of the islets in 100 μl of the wash buffer and transferred into each well of a black microtiter plate. Measurement of fluorescence intensity was performed with excitation of 485 nm and emission of 535 nm in a fluorescent plate reader (GENios, Tecan US Inc. (Durham, NC)).

Oxymetry measurements of the solutions in Islet Isolations: O_2 tension and pH were measured in the pancreas perfusion medium (PolyHeme and RPMI) for rat isolations before and after digestion phase using a blood gas analyzer (ABL/700 Radiometer, Copenhagen). A 1ml sample of the oxygenated RPMI/PolyHeme or RPMI was taken from the medium containing the perfused pancreas before introduction into the water bath for digestion, and promptly analyzed. Another 1ml sample was taken after 18 min of incubation and shaking of the tissue inside the 50 ml conical tube.

In Vivo Assessment

Islet function was assessed in vivo by transplantation under the kidney capsule of diabetic athymic nude mice (Harlan Industries, Indianapolis, Indiana). Animals were housed and surgeries performed under a laminar flow hood located in "Barrier" rooms at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC).

Mice were rendered diabetic by a single intraperitoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body wt. Diabetes was considered after two or more non-fasting blood glucose levels of >300 mg/dl from the tail vein, which generally occurred after a maximum of 36 hour post injection. Animals were anesthetized by inhalation with isoflurane using an isoflurane vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ. U.S.). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Islets from human pancreata were cultured for 2 days under standard culture conditions (CMRL-1066 supplemented with 10% human albumin, Insulin-transferrin-selenium (ITS)), without L-Glutamine. Islet grafts of 1,000 EIN from control or treatment groups were transplanted into each mouse under the left kidney capsule using a method previously described (12). In previous experiments, we determined that 1000 human islet equivalents would render normoglycemic less than 50% of diabetic nude mice (minimal mass transplant model). First, a total of 1,000 EIN are handpicked and pelleted in a 27-gage butterfly catheter attached to a 1ml syringe (Abbot Laboratories). To form the pellet, islets are centrifuged with a helicopter spinner custom-made for this size butterfly catheter (Geneva, Switzerland). After assuring that the animal was fully anesthetized and following aseptic techniques (bethadine and alcohol wipe), a 1cm incision was performed in the left lumbar flank of the animal through skin and muscle layers. The kidney was gently exposed with a sterile cotton bud; once the kidney was exposed, the needle was introduced gently below the capsule (between the capsule and the renal parenchyma), then, the plunger of the syringe was gently pressed until visualization of the islets under the kidney capsule. The needle was pulled out and the point of entrance is cauterized. Suture of the muscle and skin layers are performed separately with prolene 6-0 (Ethicon Inc.). The animals are provided with pure O_2 until full recovery and transferred to their cages with a heat lamp. Successful transplantation was defined by reduction of glycemia levels to <200 mg/dl after transplantation. After 5-7 weeks post-transplantation, normalized recipients underwent nephrectomy in order to remove the islet graft. Return to hyperglycemia was interpreted as direct proof of islet graft function rather than spontaneous recovery of the native pancreas.

Islets isolated from rat pancreata were transplanted immediately post-isolation, without previous culture period. In previous experiments, we determined that 250 ischemic rat islets would render normoglycemic less than 50% of diabetic nude mice (minimal mass model). The surgical technique and transplantation method followed the same procedure described in the previous paragraph for the human islets.

Graft function was also assessed by the lag period to achieve normoglycemia, and by intraperitoneal glucose/arginine stimulation test (IPG/AST) one week after transplantation. Briefly, for IPG/AST, a solution containing 2mg/kg body weight of glucose and 0.3 mg/kg of L-arginine was injected IP into randomly selected euglycemic animals. Bood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 minutes).

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Chapter 3

Improvement of Pancreatic Islet Isolation Outcomes Using Glutamine Perfusion During Isolation Procedure[*]

Introduction

The Edmonton group has established an islet isolation protocol that results consistently in insulin-independence after transplantation (23). Nevertheless, a limited ability to isolate large numbers of viable islets from a single pancreas has made it necessary to pool islets from several donors to achieve a critical beta cell mass (21). Islet engraftment after transplantation is often hampered by unspecific inflammation, hyperglycemia and metabolic over-demand, further reducing the chances to achieve insulin-independence with a single pancreas (8). If islet transplantation is to become an effective treatment for type-1 diabetes, there must be significant improvements in the quantity and quality of islets recovered from a single cadaveric donor pancreas (16).

Injury to both exocrine and endocrine tissue occurs during organ harvest, and further damage to the tissue occurs during islet isolation procedure. The subsequent generation of reactive oxygen species (ROS) further potentiates injury at the cellular level, eventually leading to overall poor islet quality and low yield.

A primary endogenous cellular defence mechanism is the reduction of these ROS via glutathione peroxidase. The tripeptide glutathione (GSH) is an integral component of this defence mechanism against cellular oxidative injury (5). Of particular relevance, pancreatic islets are extremely sensitive to oxidative stress; perhaps more so than other tissues due to intrinsically low GSH and glutathione peroxidase levels (7). It has been shown that the treatment of non-pancreatic tissues with exogenous glutamine supports intracellular glutathione pools; glutamine can be easily transported across the cell membrane (unlike glutathione) and is integrated into the tripeptide (6, 11).

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The incorporation of any intervention (glutamine) targeted at the pancreas must not interfere with the other organs involved in a multi-organ harvest. In a standard islet isolation procedure, the pancreatic duct is cannulated in the back table in order to administer the digestive enzymes for islet isolation. Consequently, the incorporation of intraductal administration of glutamine accompanying digestive enzyme treatment is a procedure that can easily be adapted for clinical islet isolation.

In this study, we demonstrate a positive effect of incorporating intraductal glutamine treatment on endogenous glutathione levels and on the quality and yield of purified rodent islets following a clinically relevant period of warm ischemia.

Materials and Methods

Islet Isolation. Sprague Dawley male rats (250-300 g) were used as organ donors for this study. All animals were housed according to the guidelines established by the Canadian Council on Animal Care. Groups were processed in a paired experimental design with 2 animals per group for all four groups processed on the same day; one experiment a day over a total of 9 days (n=18 per group). Pancreatic islets of Langerhans were isolated using the standard techniques of collagenase digestion (14) modified with a discontinuous Dextran gradient purification (2). Briefly, the rats were anesthetized using sodium pentobarbital (Somnitol™) and the common bile duct was cannulated. Standard (control) pancreatic harvest and treatment involved distention with Hanks Balanced Salt Solution (HBSS). Pancreata treated with glutamine were distended with HBSS plus glutamine (5 mM). In the standard procedure, immediately following exsanguination, the pancreas was procured and islets were isolated using collagenase digestion. In two experimental groups (with/without glutamine), warm ischemia (30 min) was induced by leaving the pancreas in situ after exsanguination. The abdomen was closed with two point stitches and a thermometer was placed in the abdomen in order to record temperature. Body temperature was maintained at 32 ± 2 °C. All harvested islets were purified with Dextran gradients; islets were handpicked free of exocrine and ductal tissue with a 16 gauge needle and washed with HBSS.

Experimental Groups: Pancreata were harvested and treated according to the following groups:

Group 1: control group, no glutamine treatment without warm ischemia;

Group 2: plus glutamine (5 mM in HBSS);

Group 3: plus 30 min warm ischemia;

Group 4: plus 30 min warm ischemia, plus glutamine (5 mM in HBSS).

Islet Count and Viability The outcome of isolations was assessed by final islet number, counting representative samples of islets stained with dithizone. Islets were counted in Islet Equivalents (I/E) per group and expressed in I/E per rat (9). Islet viability was assessed using a fluorescent double staining: Syto green/Ethidium bromide (26). Islets were assessed under a fluorescent microscope and a minimum of 30 islets were counted as representative for each sample. Islets that exhibited more that 75% green fluorescence were considered viable and islets showing at least 25 % orange were considered non-viable.

Glutathione Levels of reduced glutathione in islets were determined as a measurement of oxidative stress (4). After isolation, islets were immediately cultured over 24 hours to allow them to recover from islet isolation [this is also done clinically to facilitate maximal islet recovery]. Islets were cultured in RPMI media (glutamine-free) supplemented with 1% New Born Calf Serum, 4.5 mM HEPES, 45 IU/ml penicillin, 45 µg/ml Streptomycin, 22.3 mg/100ml dextrose [final concentrations] per 500 mL media. Glutathione was determined after this culture period to ensure non-interference with non-viable islets based on fluorescence of the glutathione-monochlorobimane adduct (4).

Statistical Analysis Results were analyzed using SPSS statistical package by one-way ANOVA and Levine's Test.

Results

No significant differences were found in rat age and body weight between the groups. A greater post-isolation islet yield for the groups without ischemia (Groups 1 & 2) was detected compared to tissues subjected to 30 min ischemia (Groups 3 & 4) (Figure 3-1). There was no significant beneficial effect of glutamine on islet yield post-isolation when pancreata were retrieved immediately following exsanguination [482 ± 35 I/E (Group 1) versus 548 ± 43 I/E (Group 2), $P>0.05$]. Additionally, no difference was detected in viability levels between Groups 1 & 2 [76 ± 4 % (Group 1) versus 79 ± 4 % (Group 2), $P>0.05$] (Table 3-1). However, when the pancreas was subjected to a clinically relevant period of ischemia, treatment with glutamine significantly increased both islet yield [343 ± 36 I/E (Group 4) versus 144 ± 16 I/E (Group 3), $P<0.05$] and islet viability [50 ± 4 % (Group 4) versus 26 ± 4 % (Group 3), $P<0.05$] (Table 3-1; Figures 3-1 & 3-2).

Glutamine treatment had a positive effect on glutathione (GSH) levels among non-ischemic tissues [2.92 ± 0.07 nmol/mg protein (Group 1) and 3.42 ± 0.05 nmol/mg (Group 2), $P<0.05$]. However, this relative increase in GSH levels was amplified in tissues treated with glutamine following 30 min ischemia. Glutathione levels were significantly greater in Group 4 (2.76 ± 0.04 nmol/mg) than in the corresponding non-glutamine treated group, Group 3 (1.66 ± 0.04 nmol/mg) ($P<0.05$; Figure 3-3).

Discussion

This study shows that intraductal administration of glutamine improves islet results from pancreata exposed to warm ischemia time. Our results also indicate that even a brief period of warm ischemia has deleterious effects on islet quantity and quality.

Ischemia is one of the conditions responsible for the production of oxidative stress in cells. Control of oxidative injury is of utmost concern in transplantation (12, 13). In the clinical setting, the pancreas is exposed to varying times of warm and cold ischemia prior to islet isolation (15, 24). This ischemic stress elicits biochemical responses that ultimately lead to cell death (3, 22) and involve a complex chain of events such as depletion of energy substrates and production of reactive oxygen species.

The depletion of GSH pools has been suggested to be part of the effector machinery associated with programmed cell death and has been known to accompany elevated ROS production during the apoptotic process (17). Pancreatic islets are extremely sensitive to oxidative stress primarily as a result of low endogenous glutathione peroxidase activity in addition to relatively low glutathione levels (7). Glutathione is one of the most important endogenous antioxidant mechanisms, essential for normal cell function and proliferation (1), it is a tripeptide, γ-glutamyl-cysteinyl-glycine, which does not move freely across cell membranes. Consequently, supplementing preservation solutions with glutathione has been of limited value in alleviating the problems associated with oxidative stress in organs subjected to prolonged ischemia. Therefore, providing the islets with a membrane-permeable (transportable) precursor is an excellent means of increasing the intracellular antioxidant capacity. The protective effects of glutamine against cell stress and damage have been clearly evidenced by different groups in organ preservation (18, 19, 25) and from the current data we can say that it has a direct consequence in obtaining a higher number of viable islets from a single isolation.

The underlying mechanism involved in the beneficial effect of intraductal glutamine on islet yield and viability was most likely a result of supplying an exogenous substrate for replenishing intracellular glutathione pools. Levels of GSH were significantly higher when pancreata, subjected to a clinically relevant period of ischemia, were treated with glutamine. Interestingly, GSH levels were greater in non-ischemic tissues as well, although the relative increase was not as dramatic. This finding is consistent with studies performed by other groups where the administration of glutamine has directly influenced the maintenance of intracellular GSH pools (6, 20). Similar results have been found with a variety of cell-types in culture where glutamine has protected them from injury and improved cell viability (10).

In this study we used intraductal and not systemic administration of glutamine. One obstacle to instituting a therapeutic intervention directed at one organ type during a multi-organ retrieval is interference with the other organs. The post-harvest intraductal flush with digestive enzymes could easily integrate a glutamine component. Preliminary tests in our lab have indicated that there is no interaction between collagenase and glutamine at the levels used in the current communication. Hence, no additional manipulation or multiple back-table flushes would need to be added to the existing islet isolation protocols.

The present study may have some limitations. First, we used a rat model and confirmation in the human setting is required. However, the presented results are likely to apply similarly to human tissue. Second, we limited the evaluation of islet isolation success to islet yields and viability. At the present time, these are the only two relevant parameters used in a prospective way to determine the suitability of a specific islet preparation for clinical islet transplantation.

In conclusion, the treatment of the pancreas with glutamine during islet isolation increases the antioxidant capacity of the islets to withstand oxidative injury by augmenting endogenous glutathione levels. The intraductal administration of glutamine at the time of digestive enzyme delivery into the harvested pancreas provides a simple method of improving islet yields and viability.

Table 3-1. Effects of glutamine treatment on islets after isolation.

Group	Yield (I/E per rat)	Viability (%)	Glutathione (nmol/mg protein)
1	482 ± 35	76 ± 4	2.92 ± 0.07
2	548 ± 43	79 ± 4	3.42 ± 0.05^{a}
3	144 ± 16*	26 ± 3*	1.66 ± 0.04*
4	343 ± 36*a	50 ± 4*a	$2.76 \pm 0.04^{*a}$

Effects of glutamine treatment on islet yield, viability and glutathione levels. Values shown are means ± SEM. * - statistically different from the respective non-ischemic group (Group 1 or 2); P<0.05. a –significantly different from the corresponding group treated without glutamine (Group 1 or 3); P<0.05.

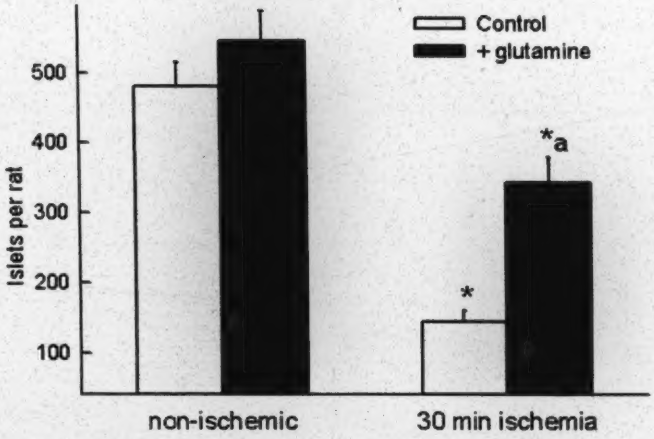

Figure 3-1. Effect of intraductal glutamine on final islet yield.

Non-ischemia groups showed higher islet recovery post-isolation than ischemia groups. Glutamine-treated pancreata (black bars) showed higher islet recovery than HBSS-treated pancreata (grey bars). * - statistically different from the respective non-ischemic group (Group 1 or 2); $p<0.05$. [a]–significantly different from the corresponding group treated without glutamine (Group 1 or 3); n= 9 isolations, $p<0.05$.

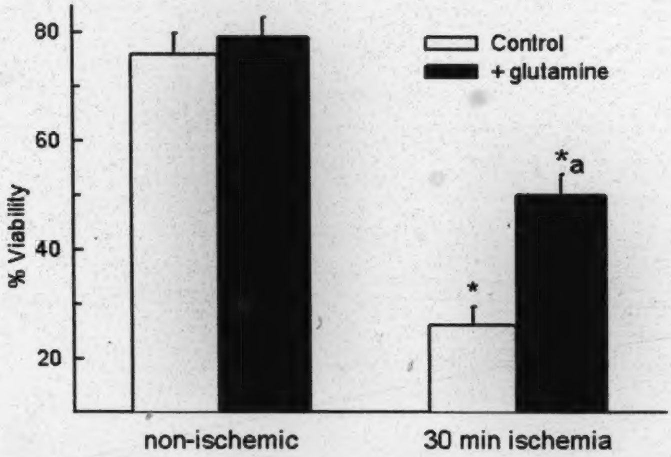

Figure 3-2. Effect of intraductal glutamine on islet viability post-isolation.

Non-ischemia groups showed higher islet viability post-isolation than the ischemia groups. Glutamine-treated pancreata (black bars) showed higher islet viability than HBSS-treated pancreata (grey bars). * - statistically different from the respective non-ischemic group (Group 1 or 2); n=9 isolations $p<0.05$. [a]–significantly different from the corresponding group treated without glutamine (Group 1 or 3); $p<0.05$.

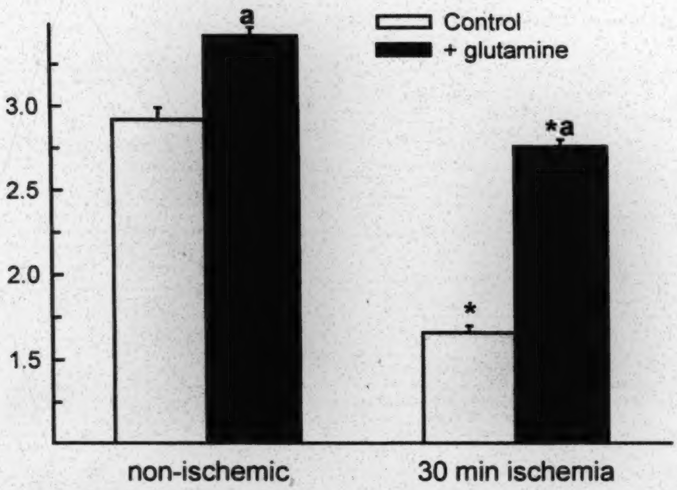

Figure 3-3. Effect of intraductal glutamine on glutathione (GSH) levels in isolated islets. Non-ischemia groups showed higher islet glutathione levels post-isolation than the ischemia groups. Glutamine-treated pancreata (black bars) showed higher islet glutathione levels than HBSS-treated pancreata (grey bars). *- statistically different from the respective non-ischemic group (Group 1 or 2); $p<0.05$. ^{a}–significantly different from the corresponding group treated without glutamine (Group 1 or 3); $P<0.05$.

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Chapter 4

The Beneficial Effect of Glutamine Perfusion During Rodent Islet Isolation on Transplantation Outcomes[*]

Introduction

After the establishment of the Edmonton Protocol, islet transplantation has re-emerged as a successful therapy for the treatment of type 1 diabetes (1). However, availability of islets continues to be one of the limiting factors from the isolation procedure that affects transplantation (2). In addition, the need for a large number of islets to achieve insulin-independence requires the use of multiple pancreata in a single patient, further increasing the demand for islets (3).

During pancreas procurement, storage and the isolation process, ischemia plays a detrimental role affecting islet yield, viability and function after transplantation (4, 5). Ischemia precedes damage produced to cellular structures by oxidative stress, which has been shown to be responsible for islet injury and loss particularly during preservation and the process of isolation (6). Pancreatic islets of Langerhans are exceptionally sensitive to oxidative stress because of the low activity of the glutathione peroxidase enzyme as well as lower reduced/oxidized Glutathione ratio (GSH/GSSG) (7). Nevertheless, there are successful ways of protecting islets from this type of injury through the use of antioxidants (8-11).

Reduced Glutathione (GSH) plays a critical role against cellular insults derived from oxidative stress. GSH is the most rapid and abundant weapon against reactive oxygen species (ROS) and regulates the redox state of many cellular substances (12).

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Moreover, GSH depletion has been shown to accompany ROS production (13), resulting in increased oxidative stress to the cells.

In a previous study, our group reported successful results after administration of intraductal antioxidants in a model of warm ischemia of rat pancreata (11). We demonstrated that the use of intraductal L-Glutamine served as a precursor for GSH in islets. This therapy protected islets from oxidative stress (higher GSH levels), further increasing islet yields and improving *in vitro* cell viability after isolation. One of the limitations of the study was the lack of *in vivo* data. Moreover, the level of oxidative injury to the islets was not assessed.

The purpose of the present study was to evaluate the effect of intraductal glutamine administration on islet transplantation outcomes in a rodent model of warm ischemia.

Materials and Methods

Islet Isolation

Male Lewis rats (Harlan Industries, Indianapolis, IN), weighing between 175-200g were used as pancreas donors for islets. All animals were housed at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC). Animals were anesthetized by inhalation with isoflurane using an isoflurane vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ. U.S.). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Rat islet isolation was performed following the standard method previously described (14), modified by using the warm ischemia model that we have developed and described (11). Briefly, after the animal was anesthetized, a laparotomy incision was performed followed by incision into the thoracic cavity and section of the heart in order to euthanise the animal by exsanguination. The abdominal cavity was closed, covered with gauze and left for 30 min before pancreas perfusion. Perfusion solution was prepared with 1 mg/ml of collagenase type XI (Sigma) reconstituted with either Hanks Balanced Salt Solution (HBSS) containing 5mM L-Glutamine (n=16 rats) or HBSS alone (Control, n=16 rats). After excision, each pancreas was placed in a 50 ml conical tube with 7.5 ml of solution followed by incubation in a 37°C water bath (digestion phase) for 18 minutes. Following digestion and wash, islets were purified from the exocrine tissue by discontinuous Ficoll density gradients (Mediatech Inc. Herndon, VA). Islets were immediately transplanted after the isolation with no culture period.

Oxidative Injury

Malondialdehyde (MDA): Lipid peroxidation was used as a marker of oxidative injury, determined by measurement of MDA, assessed by the thiobarbituric acid method (TBARS) (15). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67 % TBA, 10% phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all Sigma). 500 islets were sonicated in 700µl PBS into a cell lysate. After centrifugation at 20142 g, 500 µl of the supernatant were extracted and mixed with 875 µl of the reaction mixture, then boiled at 95-98°C for 1 hour. After this process, samples were cooled down and mixed with 750 µl of N-butanol in order to extract the MDA and avoid interference of other compounds. After a short spin, 100 µl of supernatant were extracted and read in duplicates on a 96 well plate with a fluorometer (GENios, Tecan US Inc. (Durham, NC)) set at Excitation: 530/25, Emission: 575/15. Samples were read against MDA standard (Sigma) prepared at different concentrations (2, 4, 8 mM) and expressed in nmol/mg protein.

Islet Transplantation into athymic "Nude" diabetic mice

Islet function was assessed in vivo by transplantation under the kidney capsule of diabetic athymic nude mice (Harlan Industries, Indianapolis, Indiana). Animals were housed and surgeries performed under a laminar flow hood located in "Barrier" rooms at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC).

Diabetes was induced by a single intraperitoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body wt. Diabetes was considered after three or more non-fasting blood glucose levels above 300 mg/dl from the tail vein, which generally occurred after a maximum of 72 hours post injection. Animals were anesthetized by inhalation with isoflurane using a vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ. U.S.). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Rat islets were transplanted fresh after isolation. 250 islets from Glutamine or Control treated pancreata were transplanted into each mouse under the left kidney capsule as previously described (16). We had previously determined that transplantation of 250 ischemic rat islets would reverse less than 50% of recipients. Successful transplantation was defined by reduction of glycemia to below 200 mg/dl within 5-7 weeks post-transplantation, normoglycemic recipients underwent graft-bearing nephrectomy. Return to hyperglycemia was interpreted as indirect proof of islet graft function rather than spontaneous recovery of the native pancreas.

Graft function was assessed by intraperitoneal glucose tolerance test (IPGTT) 2 weeks post-transplantation, and the lag period to achieve normoglycemia. Briefly, for IPGTT, 2mg/kg body weight of glucose in 0.5 cc was injected IP into randomly selected euglycemic animals (n=6 for Glutamine and n=3 for control). Bood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 minutes).

Results

Sixteen donor rats per group were used to assess the effects of Glutamine administration to ischemic pancreata on transplantation outcomes.

Oxidative Injury: Islet levels of MDA were measured (oxidative injury) at 24 hours post-isolation. MDA levels were found to be significantly lower in the glutamine treated islets than in the control, (2.9±0.94 nmol/mg of protein vs. 6.3±0.60 nmol/mg of protein respectively, p=0.04).

In vivo Function: We found that transplantation of glutamine treated islets was able to render normoglycemic a significantly higher number of diabetic nude mice than the control group, p=0.001. In fact, 100% of diabetic mice transplanted with glutamine treated islets achieved long-term normoglycemia, against 57% of the mice transplanted with islets from the control group (Figure 4-1). Moreover, the lag time to reach normoglycemia was shorter in mice transplanted with islets from the glutamine group than the control; (1±0 days vs. 3.5±0.9 days respectively, p=0.03). The five-week follow up of the transplanted mice from both groups showed a significantly improved regulation of blood glucose levels in the glutamine group than the control (Figure 4-2).

IPGTT were performed in randomly selected animals that reached normoglycemia (n=6 out of 8 for glutamine and n=3 out of 4 for control). Mice transplanted with islets treated with glutamine showed a normal response to the test. The glutamine group exhibited a significantly smaller area under the curve than the control, (6816±532 glycemia x minutes vs. 10135±694, respectively), p=0.007. (Figure 4-3).

Discussion

Our study shows that intraductal perfusion of ischemic pancreata with L-glutamine decreased oxidative injury to the islets and improved *in vivo* outcomes after islet transplantation.

Oxidative stress has been shown to negatively affect the outcome of islet isolation (8, 9, 11), compromising islet availability for transplantation. Moreover, a significant number of transplanted islets are lost because of cell death mediated at least in part by oxidative injury (17). ROS can injure cells by DNA strand breakage and peroxidation of proteins and lipids (18).

Our group previously reported the effectiveness of intraductal Glutamine administration in increasing GSH levels and islet isolation outcomes (11). As expected, in the current study we demonstrate that intraductal glutamine perfusion also decreased oxidative injury to the islets. This result is supported by the correlation shown between higher GSH levels with lower oxidative injury in pancreatic islets (19).

We also found improved graft function after transplantation with islets from the glutamine group determined by the higher percentage and shorter lag time of diabetic mice achieving normoglycemia. Islets are distinguished by their relatively low antioxidant enzyme content and activity, which render them susceptible to oxidative stress after transplantation. In a recent study, Robertson et al. (20) demonstrated that overexpression of antioxidant enzymes (related to GSH) can improve β-cell function in animal models.

Blood glucose levels in transplanted mice after 5 weeks follow-up were lower (normal range) and more stable for the glutamine group, whereas for the control, glycemia values maintained over 200 mg/dl after the first week. The response to an IPGTT of "cured" mice transplanted with glutamine-treated islets was improved (normal response), compared to the diabetic response of mice from the control group. These results point in favor of an improved insulin secretory capacity (as per blood glucose measurement) of glutamine islets against the control group. They also correspond with the observation that oxidative stress contributes importantly to the loss of endogenous β-cell insulin gene expression, content, and secretion. The use of GSH-promoting antioxidants like N-acetyl cysteine have shown to prevent the adverse effects of glucose toxicity via oxidative stress on β-cell function both in vivo and in vitro (21). After transplantation, the new islet graft is placed in a hyperglycemic environment, which is prone to produce oxidative stress and β-cell damage. Enhancing the antioxidant defense mechanisms in pancreatic islets may be a valuable approach for improving islet function post-transplantation.

We conclude that intraductal Glutamine perfusion decreases oxidative injury produced after ischemia during islet isolation and improves islet engraftment and function after transplantation in diabetic mice.

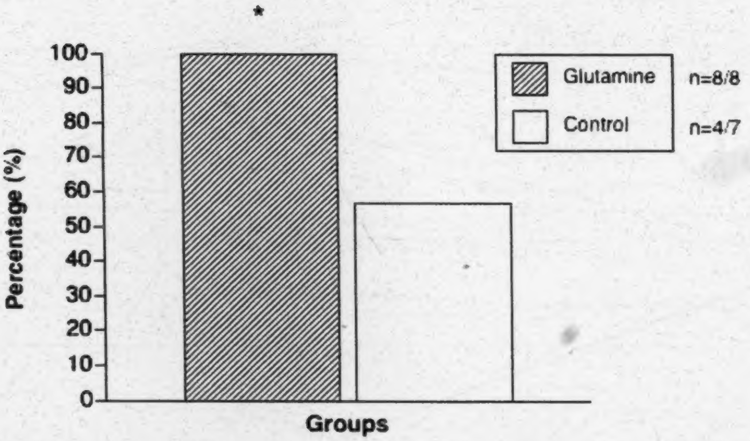

Figure 4-1. Percentage of diabetic mice achieving normoglycemia after transplantation with islets from glutamine and control groups.

Percentage of mice reaching normoglycemia after transplantation with islets from glutamine group (filled bar) was higher than mice transplanted with islets from control group (empty bar). Glutamine n=8/8 mice and Control n=4/7 mice. *p=0.001.

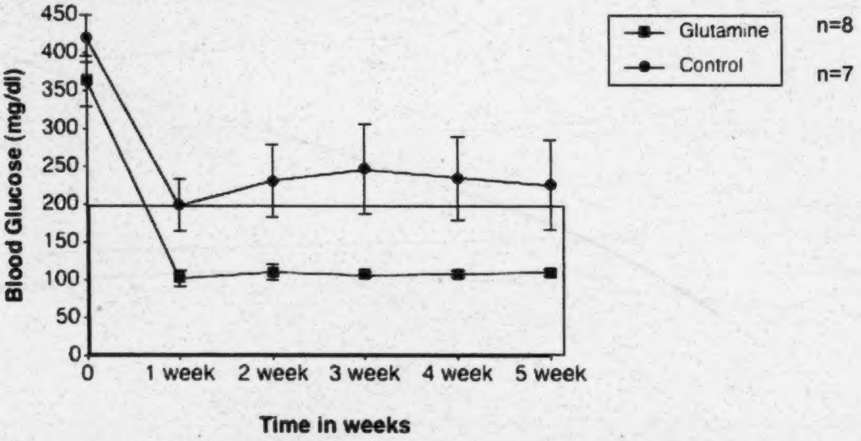

Figure 4-2. Five-week follow up of blood glucose values in all transplanted mice.

Blood glucose values are lower for mice transplanted with islets from glutamine group (square dots) than mice transplanted with islets from control group (circle dots). Glutamine n=8, Control n=7; p<0.01 for every point except pre-transplant values.

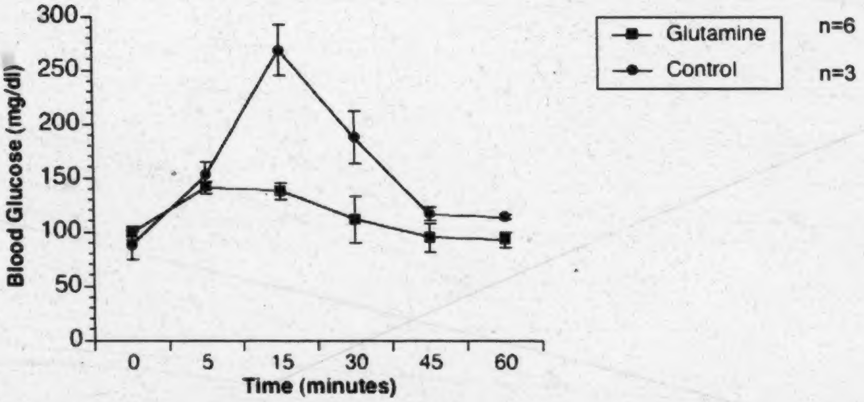

Figure 4-3. ***In Vivo*** **islet function assessment through an Intraperitoneal Glucose Tolerance Test (IPGTT)**

Mice transplanted with islets from glutamine group showed a normal response to an IP glucose challenge (square dots) compared to a diabetic response from mice transplanted with islets from the control group (circle dots). Representative sample of mice that reached normoglycemia transplanted with islets corresponding to Glutamine (n=6) and Control (n=3) groups. p=0.001.

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Chapter 5

Intraductal Glutamine Administration Reduces Oxidative Injury During Human Pancreatic Islet Isolation[*]

Introduction

In the transplant setting, islets of Langerhans are faced with various types of stress related to the isolation and transplantation procedure, which trigger a cascade of cell signaling pathways that compromise their function and viability (1-5). Moreover, variable times of ischemia, affect the number and function of islets recovered (6-8). Despite reports by groups achieving a critical beta cell mass and effective islet transplants into diabetic patients from one donor pancreas (9, 10), the availability of pancreata and sufficient islets to achieve normoglycemia in patients continues to be the Achilles heel of islet transplantation (11).

Oxidative stress plays a pivotal role in cell injury during islet isolation and transplantation procedures (12-14). The use of antioxidants has been shown to protect islets from oxidative injury during the isolation procedure and during the culture period (15-18). One of the most important non-enzymatic antioxidant defense compounds involved in this reaction is Glutathione (GSH) (19-21), a tripeptide (glutamate-glycine-cysteine) for which cell membranes are not readily permeable (22). Cells have the appropriate machinery to produce GSH if provided with its precursors. One way to increase GSH levels is to increase intracellular glutamate since this promotes ATP production, cystine uptake and alleviates the glutamate-induced inhibition of L-gamma-glutamyl-L-cysteine synthase (HCS) activity (23). Glutamine supports the intracellular pool of glutamate, avoiding its depletion and the depletion of GSH; glutamine can be considered a precursor molecule to glutathione (24). The importance of glutamine for cell survival and proliferation in vitro was first described in detail by Eagle et al in 1956 (25). A cytoprotective and antiapoptotic effect of glutamine has been demonstrated in intestinal epithelial cells by Evans et al (26), and also in pancreatic islets (27). Glutamine has also been shown to enhance islet cell function through increase in β-cell insulin secretion capacity (28-31).

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In a rodent study (18), we demonstrated that intraductal administration of L-Glutamine increases GSH levels in pancreata exposed to 30 minutes warm ischemia. We found no difference between glutamine or control groups when there was no injury to the pancreas, but when pancreata were subjected to ischemia glutamine improved viability and islet yields after isolation. Similar results with increased islet yields were reported in pigs when using intraductal glutamine administration before the islet isolation procedure (32).

The purpose of the present study was to evaluate islet isolation and transplantation outcomes after intraductal glutamine administration in human pancreata procured from marginal donors. We hypothesize that intraductal administration of L-Glutamine replenishes intracellular GSH levels, consequently protects against oxidative injury and improves human islet isolation and transplantation outcomes by preventing lipid peroxidation and oxidative stress.

We therefore, conducted a study where a group of human pancreata procured from donors deemed unsuitable for pancreas or islet transplantation were treated with glutamine through the main pancreatic duct, to determine if pancreatic glutamine perfusion improved not only islet recovery and function in vitro, but also in vivo after transplantation into diabetic athymic nude mice.

Research Design and Methods

Islet Isolation and Antioxidant Treatment

Human pancreata refused by other centers for whole pancreas or islet transplantation were obtained from organ procurement organizations (Gift of Hope, Illinois; Gift of Life, Michigan; and Indiana). Pancreata were obtained using standard organ procurement techniques (pancreas recovered with spleen and duodenum attached) by the liver team responsible and sent on ice to the Cell Isolation Laboratory at the University of Illinois at Chicago (UIC). The pancreata were perfused at the same time of liver and kidney perfusion via abdominal aorta with cold preservation solution. Human pancreata from 12 donors were used. Donor characteristics summarized in Table 5-1.

Islets of Langerhans were isolated using the semi automated method described by Ricordi et al. (33). Pancreas digestion was performed using Liberase-HI (Roche, Indianapolis, IN). The enzyme was dissolved with cold Hanks balanced salt solution (HBSS) for the control group. The enzyme for the treatment group was dissolved in HBSS containing a final concentration of 5mM L-Glutamine. Prior to digestion, pancreata were manually perfused (syringe loaded) through the main pancreatic duct with either the standard HBSS (control, n=6) or with HBSS + 5mM L-Glutamine (treatment, n=6), and left at least 15 min before its transfer to the digestion chamber. Pancreata were randomly allocated to treatment or control groups. After digestion islets were purified in a COBE 2991 cell separator using continuous Ficoll gradients and purity assessed using dithizone staining as previously described (8).

Assessment of Isolation outcomes.

In Vitro:

Islet Yield: The outcome of the islet isolation was assessed through the quantification of islet mass by dithizone staining and counts expressed in equivalent islet numbers (EIN). Two independent investigators assessed the quantity and purity of the preparations in accordance to the criteria established at the 1989 International Workshop on Islet Assessment (34).

Viability: Islet viability was assessed by fluorescent staining with Syto-Green/Ethidium Bromide (35), and the percentage of dead and live cells was estimated in both, control and treatment groups as previously described (18). Briefly, double fluorescence was performed to assess the amount of live (green) versus dead (red) islet cells in a representative sample, where a minimum of 100 islets were counted per sample.

Function: In vitro islet function was performed by static glucose incubation and expressed in terms of stimulation index (SI) by calculating insulin secretion of islets challenged with Krebs-Ringer bicarbonate buffer (KRBB) (pH 7.35) containing 10 mM HEPES and 0.5% BSA (Sigma) with high glucose concentration (16.7mM) and dividing it by insulin secretion under low glucose conditions (1.6mM) (8). Briefly, groups of 5 handpicked islets identified under a stereoscopic microscope were placed in five different wells of a 12 well-plate; then incubated with 1ml of KRBB-low glucose concentration (1.6 mM glucose final concentration) for 30 min, allowing them to stabilize insulin secretion, then the supernatant was collected and discarded. Islets were then incubated for 1 hour in low glucose KRBB (1.6 mM glucose final concentration) at 37°C and 5% CO_2, and supernatants were collected under a microscope taking care of not removing any islets from the well. The same step was repeated by addition of KRBB-high glucose solution (16.7 mM glucose final concentration) and incubation for 90 min.

Supernatants were collected and frozen at -20°C for later measurement using an ELISA kit for human insulin (Mercodia; Uppsala, Sweden).

Oxidative Stress: GSH levels were measured using the monochlorobimane (mcbm) method (36) in islets at 12 hours post-isolation as previously described (18). Briefly, 1,000 EIN were cultured in one well of a 12 well-plate in 5 ml CMRL culture medium and 10 µL of mcbm (50mM) (Molecular Probes; Eugene, OR, U.S.) and were incubated for 30 min at 37°C. Islets were collected, washed with phosphate buffered saline (PBS) at a pH of 7.5, resuspended in 500 µL of 50mM TRIS buffer containing 1mM EDTA (1mL), then sonicated. After centrifugation the supernatant was read in a fluorescence plate reader (GENios, Tecan US Inc. (Durham, NC)) with excitation set at 380 nm and emission set at 470 nm.

Oxidative injury: Lipid peroxidation was used as a marker of oxidative injury, determined by measurement of malondialdehyde (MDA). MDA is a product of lipid peroxidation. MDA levels were assessed by the Thiobarbituric acid method (TBARS) (37). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67 % TBA, 10 % phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all Sigma). 1,000 EIN were sonicated in 700µl PBS into a cell lysate. After centrifugation at 20142 g, 500 µl of the supernatant were extracted and mixed with 875 µl of the reaction mixture, then boiled at 95-98°C for 1 hour. After this process, samples were cooled down and mixed with 750 µl of N-butanol in order to extract the MDA and avoid interference of other compounds. After a short spin, 100 µl of supernatant were extracted and read in duplicates on a 96 well plate with a fluorometer (GENios, Tecan US Inc. (Durham, NC)) set at Excitation: 530/25, Emission: 575/15. Samples were read against MDA standards (Sigma) prepared at different concentrations (2, 4, 8 mM).

Apoptosis: Immunohistochemical Analysis (Detection of free 3-OH strand breaks resulting from DNA degradation by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL): In order to assess the influence of glutamine on islet cell apoptosis, islets from 6 different pancreata were fixed, sections performed and stained apoptosis. (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit, Chemicon International, USA). 5 sections from each pancreas per day (1 and 3) were stained and counted; a minumum of 30 islets per section were randomly counted by field.

In Vivo Assessment. Islet Transplantation into athymic "Nude" diabetic mice.

Islet function was assessed in vivo by transplantation under the kidney capsule of diabetic athymic (Balb/c) nude mice (Harlan Industries, Indianapolis, Indiana). Animals were housed and surgeries performed under a laminar flow hood located in "Barrier" rooms at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC).

Mice were rendered diabetic by a single Intraperitoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body wt. Diabetes was considered after two or more non-fasting blood glucose levels of >300 mg/dl from the tail vein, which generally occurred after a maximum of 36 hour post injection. Animals were anesthetized by inhalation with isoflurane using an isoflurane vaporizer with anesthetic system (Viking Medical; Medford Lakes, NJ. U.S.). This procedure was performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Islets from 12 different pancreata (control and treatment) were cultured for a maximum of 36 hours under standard culture conditions (CMRL-1066 supplemented with 10% human albumin, Insulin-transferrin-selenium (ITS)), without L-Glutamine. This was performed while giving time for mice to become diabetic. Islet grafts of 1,000 EIN from control or glutamine treated groups were transplanted into each mouse under the left kidney capsule using a method previously described (38). In previous experiments, we determined that 1000 human islet equivalents would render normoglycemic less than 50% of diabetic nude mice. Successful transplantation was defined by reduction of glycemia levels to <200 mg/dl after transplantation. After 5-7 weeks post-transplantation, normalized recipients underwent nephrectomy in order to remove the islet graft. Return to hyperglycemia was interpreted as indirect proof of islet graft function rather than spontaneous recovery of the native pancreas. Graft function was also assessed by the lag period to achieve normoglycemia, and by intraperitoneal glucose/arginine stimulation test (IPG/AST) one week after transplantation. Briefly, for IPG/AST, a solution containing 2mg/kg body weight of glucose and 0.3 mg/kg of L-arginine was injected IP into randomly selected euglycemic animals. Bood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 minutes).

Statistical methods: Statistical analysis was carried out by Student's *t* test and Pearson Chi-Square test. *P* values ≤0.05 were regarded as statistically significant.

Results

Twelve different donor pancreata were used to assess the influence of glutamine perfusion on islet isolation and transplantation outcomes. Donor characteristics in regard to age, weight and cold ischemia time were comparable (Table 5-1).

Islet Isolation Results

Islet yields from pancreata perfused with L-Glutamine were significantly higher than in control pancreata (Figure 5-1). The islet purity was 83% ±3.09 for the glutamine and 71.6% ±5.02 for the control group (p=0.097).

Islet viability assessed by Syto-Green/Ethidium Bromide immediately after the isolation procedure was similar between both groups (mean±SEM, 87% ± 3 for glutamine group vs. 82% ± 4 for the control, p=0.8. The viability between both groups at 24 h and 36 h after islet isolation showed no statistical difference either (data not shown). Assessment of in vitro islet function by static glucose incubations at 12-24 hours after islet isolation show similar stimulation indices in both groups (mean± SEM for S.I., 8.9 ± 2.8 for glutamine group versus 6.91 ± 1.5 for control, p=0.2). Stimulation indices at 48 h were also similar between both groups (data not shown).

Assessement of Oxidative Stress

Glutathione: After the isolation procedure, islets from pancreata treated with intraductal glutamine had higher glutathione levels than the control islets (Figure 5-2).

Malondialdehyde (MDA): Islet levels of MDA were measured as a marker of lipid peroxidation (oxidative injury) at 24 hours post-isolation. MDA levels were found to be significantly lower in the glutamine group (Figure 5-3).

Identification of Apoptosis

Tunel: Human islets from glutamine treated pancreata showed less apoptotic cells per islet than the control group at day 1 and day 3 post-isolation (0.63±0.04 apoptotic cells per islet for glutamine at day 1 vs. 1.88±0.14 apoptotic cells per islet for control), $p \leq 0.01$; (Figure 5-4 A to D) and (0.44±0.02 apoptotic cells per islet at day 3 for glutamine vs. 1.82±0.14 for control), $p \leq 0.01$. (Figure 5-5 A to D).

In vivo islet function after transplantation into diabetic nude mice:

Islet function: In vivo function was assessed through transplantation of glutamine treated and control islets into streptozotocin induced, diabetic, athymic nude mice. We found a higher effectiveness of glutamine treated islets to render normoglycemic diabetic nude mice than the control islets. Reversal of diabetes was achieved more frequently and the lag period to reach normoglycemia was shorter in animals transplanted with islets from the glutamine group (Figure 5-6 and 5-7). Mean glycemia in the mice transplanted with islets from the glutamine-treated pancreata was significantly lower than in the mice transplanted with the control islets (Table 5-2). Intraperitoneal glucose/arginine stimulation test was performed in randomly selected animals reaching normoglycemia from both groups. Mice transplanted with islets from pancreata treated with glutamine exhibited a normal response to the test when compared to the mice transplanted with control islets, represented by a significantly smaller area under the curve for the glutamine group than the control, mean± SEM (7153±381 vs. 12360±1587 respectively), $p=0.03$. (Figure 5-8).

Discussion

The prerequisite for successful islet transplantation is a large number of viable and functional pancreatic islets, ideally originating from a single donor. The organ donation, islet isolation and transplantation procedures expose pancreatic cells to various degrees of oxidative stress, compromising islet yields and cell viability and function. Accumulating evidence indicates that GSH has a central role in redox regulation (39). Our study indicates that intracellular GSH levels can be increased by the simple means of intraductal glutamine administration prior to the isolation procedure. Consequently, we found lower MDA levels in islets isolated from glutamine-pretreated pancreata, indicating less oxidative damage. Although we could not detect any differences in cell viability and islet function in vitro, the islets isolated from glutamine-pretreated pancreata performed significantly better than the controls after transplantation in diabetic nude mice.

The fact that islet viability in vitro did not differ between both groups, at least at 0, 24 and 48 hours following the isolation, suggests that the changes that occur at these times between the treated and non-treated islets are subtle and not detected by the vital stain combination of syto-green and ethidium bromide used. Nevertheless, islets from glutamine treated pancreata reversed diabetes not only in a larger amount of mice, but also with a shorter lag period than the control group. The improved outcome in terms of percentage of mice achieving normoglycemia may be related to higher islet cell survival after transplantation consequent to higher intracellular GSH levels and reduced oxidative pre-damage after the isolation procedure. This is supported by a fewer amount of apoptotic cells per islet observed in the glutamine treated islets than in the control. The longer lag period to reach normoglycemia and the diabetic response to the IPG/AST observed in the control group may in addition be the consequence of lower insulin secretory capacity induced by oxidative stress (40). We suggest that glutamine increases survival and β-cell mass by protecting islets from oxidative injury, which would ultimately lead to apoptosis and cell death. This finding correlates with previous studies observing a reduction in apoptotic cell death in pancreatic islets in the presence of glutamine (27, 41). Studies by Evans et al (26, 42) suggest an anti-apoptotic protection of cells distinct of glutathione, however, glutamine may have additional effects which our experiments and current literature reports have not addressed so far and therefore remain speculative.

A number of studies have investigated anti-oxidative and cytoprotective measurements to prevent islet injury during pancreatic islet isolation and transplantation. Bottino et al demonstrated that islet isolation outcomes can be improved by the catalytic antioxidant probe AEOL10150 (manganese [III] 5,10,15,20-tetrakis [1,3,-diethyl-2imidazoyl] manganese-porphyrin pentachloride [TDE-2,5-IP]) and observed reduced Nfkappa-B binding to DNA, reduced release of cytokines and chemokines, and reduced Poly(ADP-ribose) polymerase (PARP) activation in islet cells, resulting in higher survival and better insulin release. Other groups have demonstrated that intraductal administration of enzymes with preservation solutions improves islet yield and ischemic tolerance of isolated rodent and porcine islets assessed by functional success in vitro and in vivo (43-45).

Others have successfully protected islets from oxidative injury by viral transduction to upregulate antioxidant and anti-apoptotic genes (46-48). However, the applicability of viral transduction in clinical islet transplantation is currently limited. Our study is unique in that the antioxidant strategy used could be easily applied in clinical practice, as glutamine is a non-toxic, simple amino-acid.

We conclude from the work presented that early oxidative events contribute to loss in islet mass and function which can be prevented by the use of antioxidants. Glutamine exerts antioxidant properties by replenishing the pools of GSH in islets and reducing lipid peroxidation (MDA) and apoptosis. Intraductal injection of glutamine is a simple and clinical applicable means to improve human islet isolation and transplantation outcomes.

Table 5-1. Donor Characteristics

	Glutamine-treated Donors	Control (HBSS) Donors	*P* Value
Number	6	6	
Mean age (range)	51 (37-61)	53 (31-75)	p=0.74
Gender (% female)	50 % Females	33 % Females	p=0.55
Weight (kg)	90 (80-99)	82 (47-112)	p=0.45
Mean BMI (kg/m2) (range)	30 (26-32)	25.3 (18-30)	p=0.06
Cause of death	50% CNS bleed or stroke, 50% trauma	50% CNS bleed or stroke; 50% trauma	p=1.0
Non–heart beating donors	NONE	NONE	
Cold ischemia time	8.5 hr	8 hr	p=0.68
Glycemia (Admission)	202.3 (96 -353)	172.3 (101-286)	p=0.55
Maximum Glycemia	226.8 (117-290)	276.0 (115-405)	p=0.42
Insulin dose	22 IU (4-37)	21.1 IU (10-40)	p=0.83
Donors other organ transplanted:			
Kidneys	(6/6)	(5/6)	
Liver	(2/6)	(2/6)	
Heart	(0/6)	(0/6)	
Lungs	(0/6)	(0/6)	

Table 5-2. Weekly blood glucose values.

Weeks	**Glutamine** Glycemia (mg/dl)	**Control** Glycemia (mg/dl)	***P*-value**
0	351.58±26.44	422.7±15.53	=0.10
1	146.09±23.68*	300.9±27.26	<0.01
2	151.38±38.76*	319.9±32.37	<0.01
3	137.79±29.07*	343±39.25	<0.01
4	140.93±33.47*	333.2±39.25	<0.01
5	140.26±31.87*	322.2±40.08	<0.01

Results of weekly blood glucose values (mg/dl) of mice transplanted with islets from both groups (glutamine and control). Values are means±SEM. (*P*- values calculated by Student's t test). *p<0.01

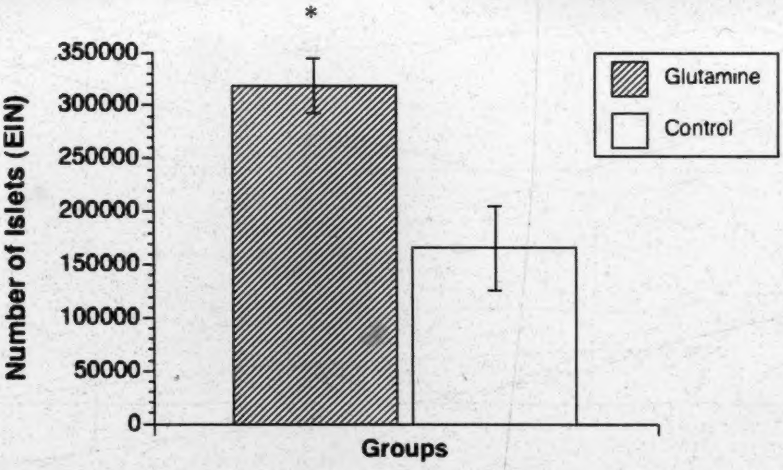

Figure 5-1. Final islet yield recovered post-isolation.

The number of islets (expressed in EIN) recovered post-isolation was twice higher for the glutamine group than for the control. Glutamine n=6 and Control n=6 human isolations. * p<0.01.

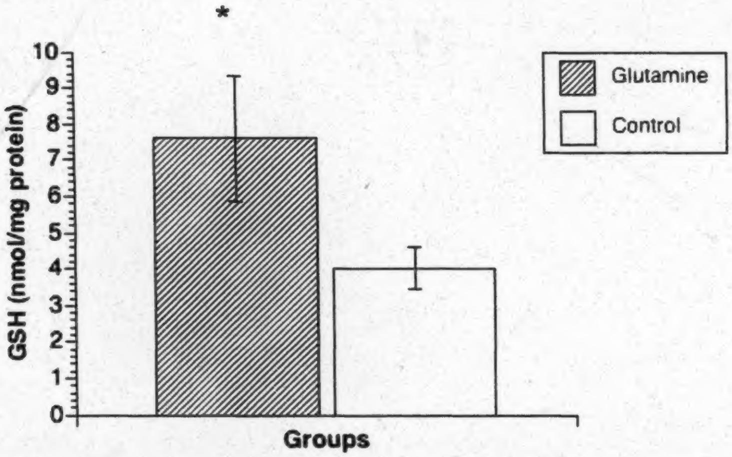

Figure 5-2. Effect of pancreatic intraductal glutamine administration on glutathione (GSH) levels in isolated human islets.

The levels of GSH (nmol/mg of total protein) were higher for islets from the glutamine group than the control. Glutamine n=6 and Control n=6 human pancreata. *p≤0.05.

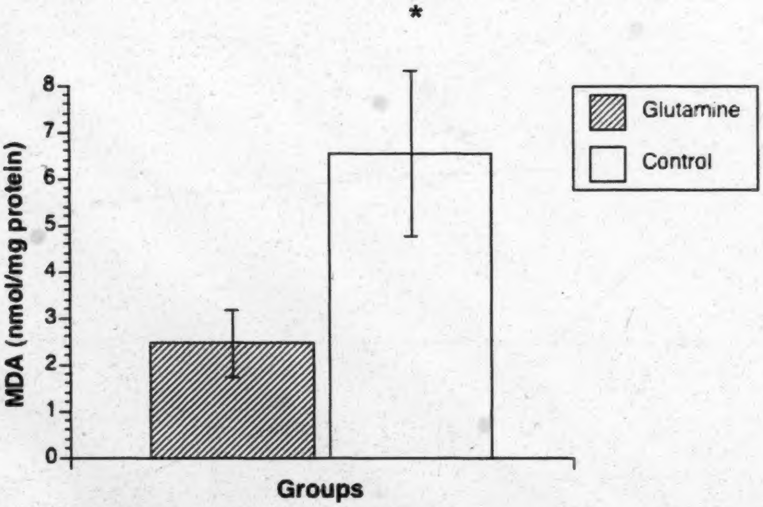

Figure 5-3. Effect of pancreatic intraductal glutamine administration on levels malondialdehyde (MDA) in isolated human islets.

The levels of MDA (nmol/mg of total protein) were higher for islets from the glutamine group than the control. Glutamine n=6 and Control n=6 human pancreata. *p≤0.05.

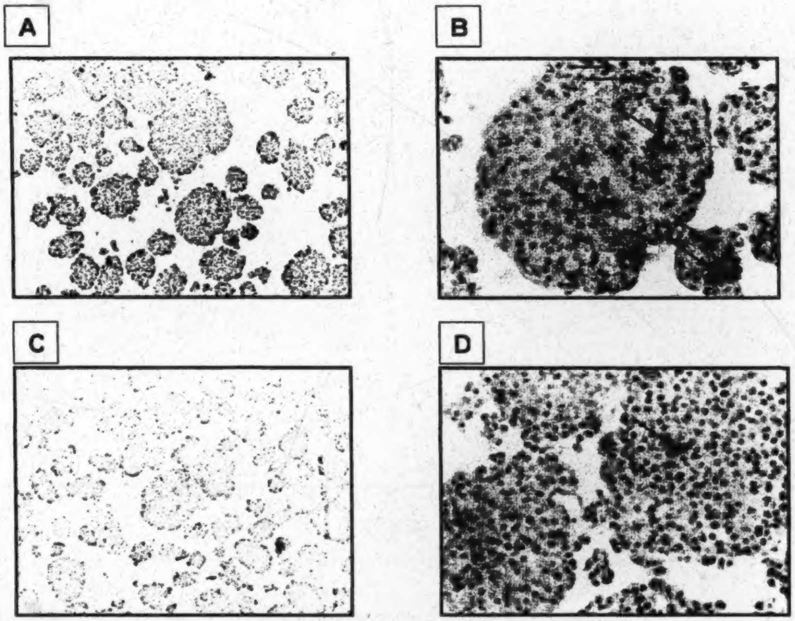

Figure 5-4. Islet Cell Apoptosis 1 Day after isolation.

(A) Control islet section at 10x magnification showing dark brown dots corresponding to apoptotic islet cells. (B) Control islet section at 40x magnification showing apoptotic islet cells (black arrows). (C) Glutamine treated islet section at 10x magnification showing fewer dark brown dots, corresponding to apoptotic islet cells. (D) Glutamine treated islet section at 40x magnification showing fewer apoptotic islet cells (black arrow).

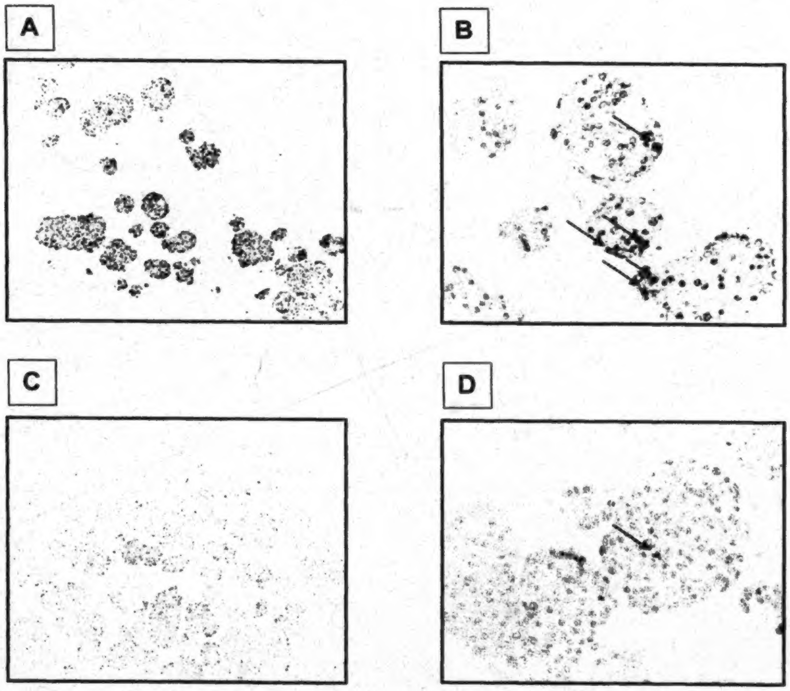

Figure 5-5 Islet Cell Apoptosis 3 days after isolation.

(A) Control islet section at 10x magnification showing dark brown dots corresponding to apoptotic islet cells. (B) Control islet section at 40x magnification showing apoptotic islet cells (black arrows). (C) Glutamine treated islet section at 10x magnification showing fewer dark brown dots, corresponding to apoptotic islet cells. (D) Glutamine treated islet section at 40x magnification showing fewer apoptotic islet cells (black arrow).

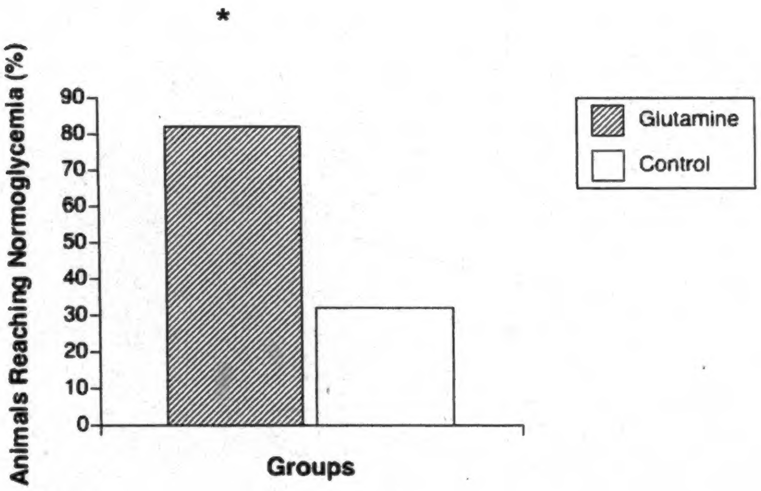

Figure 5-6. Number of mice reaching normoglycemia after transplantation with islets from glutamine and control groups.

The percentage of mice reaching normoglycemia after transplantation with islets from the glutamine group (filled bar) was higher than mice transplanted with islets from the control group (empty bar). Glutamine n=10/12 mice and Control n=6/23 mice. * p<0.01.

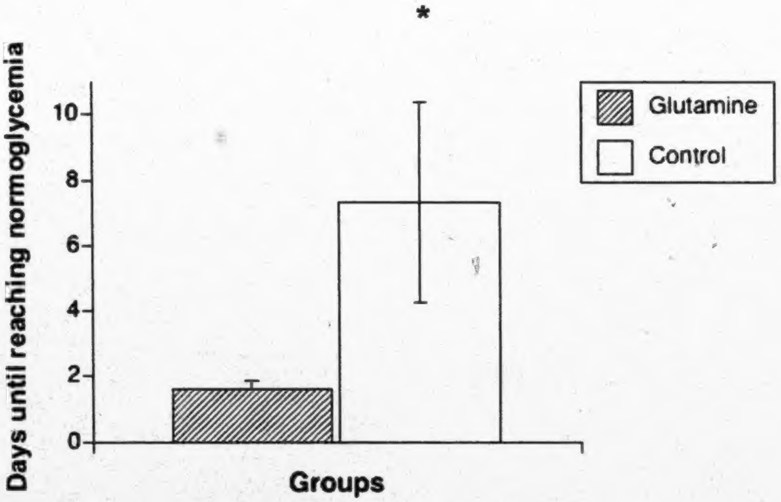

Figure 5-7. Number of days (lag time) to reach normoglycemia for mice transplanted with islets from glutamine and control groups.

The number of days to reach normoglycemia were less for mice transplanted with islets from the glutamine group (filled bar) than for mice transplanted with islets from the control group (empty bar). Glutamine n=10 mice and Control n=6 mice. *p<0.01.

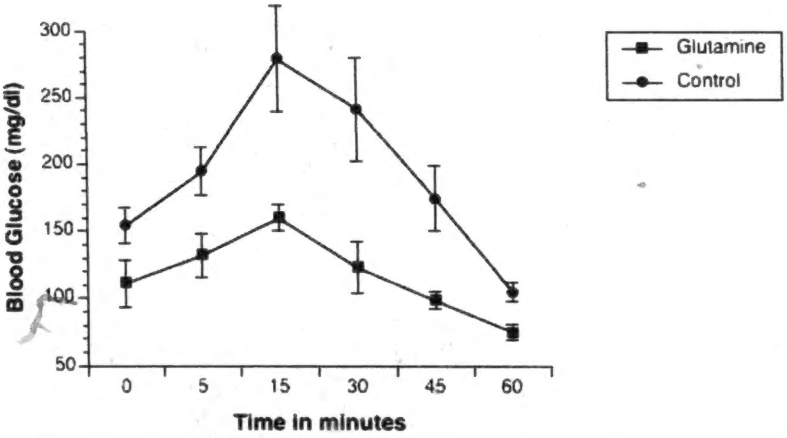

Figure 5-8. Intraperitoneal Glucose/Arginine Stimulation Test (IPG/AST) in mice transplanted with islets from glutamine and control groups.

Mice transplanted with islets from glutamine group showed a normal response to an IP glucose/arginine challenge (square dots) compared to a diabetic response from mice transplanted with islets from the control group (circle dots). A representative sample of normoglycemic mice transplanted with islets corresponding to Glutamine (n=4) and Control (n=4) groups was used. p=0.001.

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Improved Outcomes in Islet Isolation and Transplantation by the Use of a Hemoglobin-based O_2 Carrier*

Introduction

Islet transplantation can reverse insulin-dependent diabetes (1-3), but the procedure is still hampered by a short supply of islets and a gradual loss of islet function after transplantation (4). The inconsistency of islet isolation outcomes has been a major limitation to widespread clinical application of islet transplantation. Among the variety of factors influencing post-isolation islet yield, viability and function, ischemia time is of particular importance. The length of ischemia is inversely correlated with islet isolation outcomes (5-8). Ischemia renders cells more susceptible to oxidative stress by impairing mitochondrial antioxidant defenses (9). Providing O_2 to ischemic tissue has been shown to be a "double edged sword" due to reperfusion injury (10-13). Reactive Oxygen Species (ROS) produced by mitochondria play a significant part in this type of injury. Oxidative stress to pancreatic islets during the isolation procedure has been well documented (14-16), and the use of antioxidants has been shown to protect islets from oxidative injury (17-20). Organ preservation solutions such as histidine-tryptophan-ketoglutarate (HTK) and University of Wisconsin (UW) solution (21-23) are designed to protect pancreatic tissue from the deleterious effects of ischemia, but do not prevent ischemia per se.

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Maintaining an appropriate O_2 level would seem important to prevent ischemic damage and reperfusion injury during organ preservation and pancreatic islet isolation. Indeed, artificial oxygen carriers, such as perfluorocarbons (PFC), have a beneficial effect on islet isolation and transplantation outcomes when used during pancreas preservation with UW solution in the two layer method (TLM) (24-27).

Artificial oxygen carriers are synthetic solutions capable of binding, transporting and unloading O_2. Artificial oxygen carriers have been originally developed as blood substitutes, but none of the PFC based products have been approved for clinical use, and in clinical trials anaphylactic reactions were observed (28). Moreover, PFCs have the inconvenience of being hydrophobic and difficult to keep in aqueous solution (29). Hemoglobin-based O_2 carriers (HBOC's) are hydrosoluble, and in phase I and II trials no anaphylactic reactions have been observed (30-32). In contrast to PFC, Polyheme presents with an O_2 saturation curve similar to red blood cells.

We hypothesize that intraductal pancreas perfusion with a HBOC provides sufficient oxygen during the islet isolation procedure to reduce ischemia and improve post-isolation islet viability and function. The purpose of the present study was to test this hypothesis in a rodent model of islet isolation and transplantation.

Research Design and Methods

Preparation of PolyHeme Solution

Polymerized human hemoglobin was manufactured and kindly donated by Northfield Industries, Evanston, Illinois. PolyHeme containing 10 g/dl of polymerized hemoglobin was custom prepared for our isolation model. We investigated the effect of collagenase on the stability of polymerized hemoglobin by HPLC analysis. PolyHeme was incubated with or without collagenase under different conditions, before and after oxygenation; at 4° and 37° C. HPLC analysis did not reveal any degradation of Polyheme. In addition, we studied the formation of Methemoglobin (MetHb) and carboxyhemoglobin (COHb) after various oxygenation times and did not find any significant MetHb or COHb formation.

250 ml of PolyHeme solution were packed sterile bags (500 ml capacity) to allow for the introduction of 250 ml of O_2. In previous tests it was determined that PolyHeme had a shelf life of 12 months. PolyHeme was stored at 4°C for no more than 9 months.

Islet Isolation and Preservation Treatment

All animals were housed at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC) and procedures involving animals were performed in accordance with the guidelines of the National Institutes of Health and the Animal Care Committee (ACC) at UIC. Male Lewis rats (Harlan Industries, Indianapolis, IN), weighing between 175-200g were used as pancreas donors for islets. Animals were anesthetized by isoflurane inhalation using a vaporizer and masks (Viking Medical; Medford Lakes, NJ.). We had 2 experimental groups:

PolyHeme Group: PolyHeme containing collagenase: n=10 islet isolations

Control Group: RPMI-1640 containing collagenase: n= 10 islet isolations

Rat islet isolation was performed following the standard method previously described (33), modified by using the warm ischemia model that we have developed and described (20). Briefly, after the animal was anesthetized, a laparotomy incision was performed followed by incision into the thoracic cavity and section of the heart for euthanasia by exsanguination. The abdominal cavity was closed, covered with gauze and left for 30 min before pancreas perfusion. 1 mg/ml of collagenase type XI (Sigma) was reconstituted either with PolyHeme/RPMI (Treatment) or RPMI 1640 (Control), and both, treatment and control were oxygenated by bubbling the solutions with 100% O_2 for 15 minutes. The oxygenated enzyme solutions were injected via the bile duct and into the main pancreatic duct for distention of the pancreas. After excision, each pancreas was placed in a 50 ml conical tube with 7.5 ml of its respective perfusion solution followed by incubation in a 37°C water bath (digestion phase) for 18 minutes. After this step, each pancreas was gently shaken in the tubes, washed with cold RPMI and transferred into a 500 ml beaker. After wash, islets were purified from the exocrine tissue by discontinuous Ficoll density gradients (Mediatech Inc. Herndon, VA). Islets were centrifuged for 15 min at 640 g and then handpicked from the middle layer of the Ficoll gradient, followed by washing and culture in RPMI containing 10% fetal calf serum (FBS), 10% Penicillin/Streptomycin (Invitrogen) without glutamine, for 24 hours culture at 37°C.

Oxymetry Assessment: O_2 tension and pH were measured in the pancreas perfusion medium (PolyHeme and control) before and after digestion phase using a blood gas analyzer (ABL/700 Radiometer, Copenhagen, Denmark).

In Vitro evaluation

Islet Yield: Dithizone stained islets from a representative sample were counted under a stereoscopic microscope (Leica Microsystems, Bannockburn, IL).

Vital staining: Islet viability was assessed by staining with trypan blue dye (Sigma). Islets stained on more than 25% of their surface were considered dead. Live versus dead islets were assessed in a representative sample, where a minimum of 50 islets were counted per sample.

Apoptosis: The level of apoptotic cell death was measured using a living cell fluorescein active caspase-3 staining kit (Biovision, California). Briefly, an aliquot of 1,200 islets per group was counted and divided into 4 eppendorf tubes with 300 μl of media (RPMI 1640 with 10% FBS and 10% Pen/Strep). 1 μl of FITC-DEVD-FMK (fluorescent dye for Caspase-3) was added into 2 of the tubes of each group (the other 2 were left without the probe as controls for the assay) and incubated for 1 hour at 37°C and 5% CO_2. Cells were centrifuged at 344 g for 1 min and supernatant removed. Cells were then resuspended with the wash buffer from the kit, repeating this step twice, followed by resuspension of the cells in 100 μl of the wash buffer and transferred into each well of a black microtiter plate. Measurement of fluorescence intensity was performed with excitation of 485 nm and emission of 535 nm in a fluorescent plate reader (GENios, Tecan US Inc., Durham, NC).

Intracellular Ca^{2+} measurement: Intracellular Ca^{2+} during glucose stimulation was measured for functional evaluation of isolated islets, using standard wide-field fluorescence imaging with dual-wavelength excitation fluorescent microscopy (34, 35). Briefly, islets attached to glass coverslips were loaded with Fura-2/AM, a ratio-metric Ca^{2+} indicator (5 μM; Molecular Probes Inc., Eugene, OR) and incubated for 30-min at 37°C in Krebs (2 mM glucose). The coverslip was mounted in a microperifusion chamber (Medical Systems Inc, Paola, KA) on the specimen stage of an inverted microscope equipped for epifluorescence (TE-2000U, Nikon Inc, Melville, NY.) and perifused by a continuous flow (rate 2.5 ml/min) of 5% CO_2-bubbled Krebs buffer at 37°C (pH 7.4).

Krebs containing different glucose concentrations (5, 8, and 14 mM) was administered to the islets and followed for 15 min each, rinsing with Krebs (2 mM) in between. Single islets were visualized with a 40x oil-immersion quartz objective and multiple islets simultaneously imaged with 10x-20x objectives. Intracellular free Ca^{2+} concentration after glucose stimulation was measured using a fluorescent video charge-coupled device camera (Roper Cascade CCD) and expressed as a ratio of fluorescence intensity of Fura-2 dual-wavelength excitation at 340 and 380 nm. Detection of fluorescence emission at 510 nm was performed using Metafluor software (Universal Imaging, Downingtown, PA). Estimation of the basal level of Ca^{2+} was accomplished by measuring pre-stimulation levels. The percentage change of intracellular Ca^{2+} between both groups was calculated by the maximum increase after glucose stimulation, minus the basal (2mM glucose) Ca^{2+} level for each group.

Addition of Tolbutamide*:* Tolbutamide, an inhibitor of K^+-ATP channels (100 µM concentration) was added after 5 minutes to the perifusion medium containing islets in Krebs (2 mM glucose). The measurement of intracellular Ca^{2+} levels after the addition of tolbutamide was performed in islets as described above, but without glucose stimulation.

Static glucose incubation: Static glucose incubation was used to compare glucose induced insulin secretion (stimulation index, SI) between the groups. SI was defined by the ratio of stimulated versus basal insulin secretion (36). Briefly, for each experiment, groups of 5 handpicked islets with similar size (approximately 100 µm) were placed in five different wells of a 12 well-plate (5 replicates); then pre-incubated with 1 ml of Krebs-low glucose concentration (1.6 mM glucose final concentration) for 30 min, then the supernatant was collected and discarded. Islets were then incubated for 1 hour in low glucose Krebs (1.6 mM glucose final concentration) at 37° C and 5% CO_2, and supernatants were collected under a microscope taking care of not removing any islets from the well. The same step was repeated by addition of Krebs-high glucose solution

(16.7 mM glucose final concentration) and incubation for 90 min. Supernatants were collected and frozen at -20°C for later measurement using an ELISA kit for rat insulin (Mercodia, Uppsala, Sweden). All samples are measured in duplicates in the kit.

Mitochondrial membrane potential and morphology: The fluorescent dye Rhodamine 123 (Rh123) is a probe of the transmembrane potential and is accumulated within the inner mitochondrial membrane (Rh123 is a lipophilic cation that integrates selectively into the negatively-charged mitochondrial membranes). In cells pre-loaded with Rh123, when membrane potential increases (hyper-polarization) as seen after glucose stimulation, more Rh123 is concentrated into the mitochondrial membrane, leading to aggregation of dye molecules and decrease (quenching) of the fluorescence signal. Rh123 was used as previously described (37). Briefly, islets were incubated in Krebs solution with glucose (2mM), supplemented with 10 µg/mL Rh123 (Molecular Probes, Eugene, OR) for 20 min at 37°C, then placed into a temperature-controlled perfusion chamber (Medical Systems Inc, Paola, KS) mounted on an inverted epifluorescence microscope (TE-2000U, Nikon Inc, Melville, NY.) The islets were perifused by a continuous flow (rate 2.5 ml/min) of 5% CO_2-bubbled Krebs buffer at 37°C (pH 7.4). Islets were then stimulated with 14 mM glucose and the changes in fluorescence were measured for 15 min after stimulation. Rh123 fluorescence was excited at 540 nm and emission measured at 590 nm. Images were collected with a charged coupled device camera (Roper Cascade CCD). Data was normalized to the average fluorescence intensity recorded during a five-minute period prior to glucose stimulation. All experiments were performed at 37°C. The percentage change in fluorescence intensity between both groups was calculated by the maximum reduction in fluorescence intensity after 14 mM glucose stimulation, minus the basal fluorescence intensity for each group.

For mitochondrial morphology assessment islets from PolyHeme and control groups were incubated with Rhodamine 123 in Krebs buffer at 2.5 μM for 15 minutes and visualized using a Carl Zeiss LSM 510 confocal microscopy equipped with 60 X water immersion objective. The 488 nm line from an argon-krypton laser used for excitation and the emission from Rh 123 were detected through an LP 505 filter. The intensity and the distribution of fluorescence were used to morphologically characterize the mitochondrial integrity.

Oxidative Stress: GSH levels were measured as a marker of oxidative stress by using the monochlorobimane (mcbm) method (38) in islets at 12 hours post-isolation as previously described (20). Briefly, 500 islets were cultured in one well of a 12 well-plate in 5 ml CMRL culture medium and 10 μL of mcbm (50 mM) (Molecular Probes, Eugene, OR, U.S.) and were incubated for 30 min at 37°C. Islets were collected, washed with phosphate buffered saline (PBS) at a pH of 7.5, resuspended in 500 μL of 50 mM TRIS buffer containing 1 mM EDTA (1 mL), then sonicated. After centrifugation the supernatant was read in a fluorescence plate reader (GENios, Tecan US Inc., Durham, NC) with excitation set at 380 nm and emission set at 470 nm.

Oxidative injury: Lipid peroxidation was used as a marker of oxidative injury, determined by measurement of malondialdehyde (MDA), a product of lipid peroxidation. MDA levels were assessed by the thiobarbituric acid method (TBARS) (39). Briefly, a reaction mixture was prepared containing 0.1 M HCl, 0.67 % TBA, 10 % phosphotungstic acid and 7% sodium dodecylsulphate (SDS) (all Sigma). 500 islets were sonicated in 700 μl PBS into a cell lysate. After centrifugation at 20142 g, 500 μl of the supernatant were extracted and mixed with 875 μl of the reaction mixture, then boiled at 95-98°C for 1 hour. After this process, samples were cooled down and mixed with 750 μl of N-butanol in order to extract the MDA and avoid interference of other compounds. After a short spin, 100 μl of supernatant were extracted and read in duplicates on a 96 well plate with a fluorometer (GENios, Tecan US Inc. Durham, NC) set at Excitation: 530/25, Emission: 575/15. Samples were read against MDA standards (Sigma) prepared at different concentrations (2, 4, 8 mM).

In Vivo Assessment

__Islet Transplantation into athymic “nude” diabetic mice__: Islet function was assessed *in vivo* by transplantation under the kidney capsule of diabetic athymic nude mice (Harlan Industries, Indianapolis, Indiana). Animals were housed and surgeries performed under a laminar flow hood located in “barrier” rooms at the Biologic Resources Laboratory, University of Illinois at Chicago (UIC).

Diabetes was induced by a single intraperitoneal (IP) injection of Streptozotocin (Sigma) 220 mg/kg body weight. Diabetes was considered after three or more non-fasting blood glucose levels of >300 mg/dl from the tail vein, which generally occurred after a maximum of 72 hours post injection. Animals were anesthetized by isoflurane inhalation using a vaporizer and masks (Viking Medical; Medford Lakes, NJ. U.S.). Rat islets were transplanted without culture fresh after isolation. 250 islets from PolyHeme or control treated pancreata were transplanted into each mouse under the left kidney capsule as previously described (40). The ischemia model was established and we determined that transplantation of 250 ischemic rat islets would reverse diabetes in less than 50% of recipients. Successful transplantation was defined by reduction of glycemia to below 200 mg/dl. 5-7 weeks post-transplantation, normoglycemic recipients underwent graft-bearing nephrectomy. Return to hyperglycemia was interpreted as proof of islet graft function rather than spontaneous recovery of the native pancreas.

Graft function was also assessed by the lag period to achieve normoglycemia, and Intraperitoneal Glucose/Arginine tolerance test (IPG/ATT) 1 week post-transplantation. Briefly, for IPG/ATT, 2 mg/kg body weight of glucose and 3 mg/kg arginine in 0.5 cc was injected IP into a representative sample of randomly selected euglycemic animals (n=5 for PolyHeme and n=3 for control; in the control group only 4 animals achieved normoglycemia). Blood glucose levels were detected by tail puncture at serial time-points (0, 5, 15, 30, 45 and 60 minutes).

Statistical methods. Statistical analysis was carried out by Student's *t* test and Pearson Chi-Square test. *P* values <0.05 were regarded as statistically significant.

Results

Influence of collagenase activity on PolyHeme

To exclude enzymatic digestion of polymerized hemoglobin by collagenase, HPLC analysis were performed. The percentage of distribution of Hb tetramers in PolyHeme exposed to collagenase under different conditions (oxygenation and temperature) was measured for each group. We did not observe any significant effect of collagenase activity on PolyHeme stability (data not shown).

Oxymetry and pH assessment

Oxygen tension and pH were measured before and after the digestion phase. As expected, O_2 tension was higher in PolyHeme compared to the control in the perfusion solution (containing distended pancreata) before the digestion phase (Table 6-1). Moreover, Polyheme maintained the pH in physiological range, whereas in the control group the pH fell significantly during the digestion phase (Table 6-1).

In Vitro Evaluation

Islet isolation outcomes in PolyHeme and control pancreata were analyzed by the following parameters.

Islet Yield and Viability

Perfusion of rat pancreata with PolyHeme did not have a significant impact on post-isolation islet yields when compared to the control group (207±33 vs. 172±32 islets/rat respectively, p=0.46. However, islet viability, as assessed by vital staining, was significantly improved in the PolyHeme group (Figure 6-1).

Apoptosis

Isolated islets from PolyHeme perfused pancreata showed less apoptosis compared to the control determined by lower levels of caspase-3 (Figure 6-2).

Intracellular Ca^{2+} Levels

PolyHeme also improved islet responsiveness to glucose as determined by increased intracellular Ca^{2+} levels in islets after stimulation with different glucose concentrations (Figure 6-3 A). In all three concentrations of glucose (5, 8, and 14 mM) the PolyHeme islets demonstrated in a dose-response manner significantly higher intracellular Ca^{2+} values than the control (Figure 6-3 B). The addition of Tolbutamide (inhibitor of ATP-dependent K^+ channels) showed that when the mitochondrial ATP regulation in these channels was bypassed, there was no significant difference between both groups in intracellular Ca^{2+} levels (Figure 6-4 A and B).

Static glucose incubation

The pre-treatment of rat pancreata with PolyHeme increased significantly insulin secretion of isolated islets in response to glucose compared to the control group (Figure 6-5).

Mitochondrial membrane potential and morphology

Measurements of mitochondrial membrane potential indicated a better functional integrity of Polyheme islets than in the control group assessed by an increased percentage of change in fluorescence, (Rh123) representative of electrochemical potential as a response to glucose stimulation (14 mM), (Figure 6-6 A and B). In addition, the results of morphology assessment show mitochondria in islets from the control group appearing swollen and fragmented, assessed by decreased staining with Rh123 around the nuclei with loss of the continuity of the staining. However, PolyHeme treatment showed improved islet mitochondrial morphology with reduced swelling and fragmentation with increased staining around the nuclei (Figure 6-7).

Oxidative stress and oxidative injury

To evaluate whether O_2 delivery by PolyHeme would lead to increased oxidative stress or injury we measured GSH and MDA levels.

Oxygenated PolyHeme did not decrease glutathione levels (7.1±2.9 nmol/mg protein for PolyHeme and 6.8±2.4 for control; p=0.93). Lipid peroxidation as measured by MDA levels was not significantly different between Polyheme and control group (1.8±0.9 nmol/mg protein vs. 6.2±2.4, respectively; p=0.19) indicating the there was no increased oxidative stress by the presence of higher O_2 levels.

In vivo islet function after transplantation into diabetic nude mice

Islet function *in vivo* was assessed through transplantation of PolyHeme treated and control islets into streptozotocin induced, diabetic, athymic nude mice. The percentage of cured mice transplanted with PolyHeme or control islets was similar (6 out of 10 and 4 out of 9 respectively, p=0.4). Mice transplanted with islets treated with PolyHeme achieved normoglycemia and reversed diabetes in a significantly shorter time than the mice transplanted with islets from the control group (Figure 6-8). Moreover, the mice receiving PolyHeme islets showed better graft function with lower glucose levels during IPG/ATT (Figure 6-9).

Discussion

Our study shows that intraductal perfusion of ischemic pancreata with PolyHeme improved islet viability and function through the maintenance of mitochondrial integrity and did not lead to increased oxidative stress in isolated islets.

Mitochondria are both the "powerhouse" of the cell and major contributor to apoptotic cell death via release of cytochrome c. Improvement of mitochondrial function and integrity should lead to both improved glucose-stimulated insulin secretion and decreased cell death. Oxygen is an essential substrate for the production of energy by the cell. However, the provision of O_2 in an already damaged mitochondrial system may increase the production of reactive oxygen species (ROS) or free radicals producing further damage by oxidative stress, which is part of entity known as reperfusion injury (9). Islets are exposed to significant oxidative stress during the islet isolation and transplantation procedure (14-16).

In our study, higher O_2 availability to PolyHeme-treated islets was shown by higher O_2 tensions in the perfusion media compared to the control. The availability of substrate for mitochondria may be responsible for the improved viability observed in islets from the PolyHeme group in our study. These data are supported by the higher post isolation islet viability observed by other groups when using oxygenated PFC's during organ preservation (24-27, 36, 41).

In our study, increased O_2 availability protected PolyHeme islets from apoptosis determined by lower levels of caspase-3 than in the control group. This result was not unexpected, since hypoxia has been shown to initiate apoptosis, mainly through the release of mitochondrial mediators into the cytosol (42). An event in mitochondrial respiration is the transmembrane transfer of protons, creating an electrochemical proton gradient, which can be used as an indicator of mitochondrial viability and function. In this context, mitochondrial functional integrity was shown to be superior in PolyHeme islets with improved membrane electrochemical potential in response to glucose stimulation. Functional integrity was complemented by the conservation of mitochondrial structure in the PolyHeme islets, determined by less swelling and more elongated mitochondria. We also showed enhanced mitochondrial staining representative of improved perinuclear localization in the PolyHeme islets. Similar mitochondrial morphology was observed by Bindokas et al comparing islets from Zucker lean and diabetic fatty rats (43). In their study, structural damage was shown in the islets from the fatty rats.

Our study also indicates that *in vitro* function of isolated islets was improved by intraductal administration of PolyHeme to the ischemic pancreas. Higher stimulation indices were obtained in PolyHeme-treated islets compared to the control in response to a static glucose challenge. The enhanced function for PolyHeme treated islets was supported by higher intracellular Ca^{2+} levels in response to glucose. This test represents the efficiency of the mitochondrial pathway in increasing cytosolic Ca^{2+}, necessary for insulin secretion in beta cells. After stimulation of pancreatic islets with glucose, it is taken up into the cytosol by membrane transporters and converted into pyruvate, which enters mitochondria. Healthy mitochondria further oxidize pyruvate in order to produce energy. An increase in [ATP/ADP] ratio closes ATP-dependent potassium channels in the cell membrane. This produces depolarization, opening the Ca^{2+} channels and promoting the fusion of granules with the cell membrane during exocytosis. To further dissect the involvement of the mitochondrial pathway on insulin secretion in response to glucose we used tolbutamide (a K^+-ATP channel inhibitor). Blocking K^+-ATP channels with tolbutamide allows cells to depolarize and raise calcium levels, directly promoting insulin secretion. After the addition of tolbutamide, intracellular Ca^{2+} response to glucose was similar between both groups. These results suggest that the provision of O_2 by PolyHeme protected the mitochondrial pathway in the process of insulin secretion in response to glucose.

Long periods of ischemia can significantly alter the electron transport complexes in mitochondria. All of the complexes show a reduced activity with structural damage to the subunits after 60 min of warm ischemia (9). Damage of the electron transport chain complex results in a greater leak of electrons. Oxygen reacts with the leaking electrons to generate ROS leading to oxidative stress and tissue damage (9). In our study, the introduction of O_2 by PolyHeme did not increase oxidative stress or damage to the islets, determined by similar levels of GSH and MDA in both groups. The amount of O_2 provided by PolyHeme was more important in terms of structural and functional recovery of mitochondria, and did not produce significant amount of oxidative stress.

PolyHeme perfusion of the ischemic rat pancreas improved islet graft function *in vivo* as shown by a better response to IPG/AT stress test and a shortened lag time to reach normoglycemia after transplantation. These *in vivo* results confirmed the improved function of PolyHeme treated islets observed *in vitro*. In current studies, we are evaluating PolyHeme for human islet isolation.

In conclusion, we have shown that intraductal perfusion with PolyHeme can improve islet isolation and transplantation outcomes through the maintenance of mitochondrial integrity, and does not lead to increased oxidative stress.

Table 6-1. Oxymetry values (O_2 and pH)

	O_2 Tension (mmHg) Pre-digestion	O_2 Tension (mmHg) Post-digestion	pH with O_2 (Initial)	pH Pre-digestion	pH Post-digestion
PolyHeme	381.7±35.3*	184.3±39.8	7.4±0.03	7.4±0.03**	7.2±0.06†
Control	202.3±28.2*	128.3±27.8	7.8±0.01	6.9±0.04**	6.6±0.11†

O_2 and pH values for perfusion media (PolyHeme and Control) before and after digestion. Values are means±SEM, n=12 rats per group. *p=0.01; **p=0.001; †p=0.009.

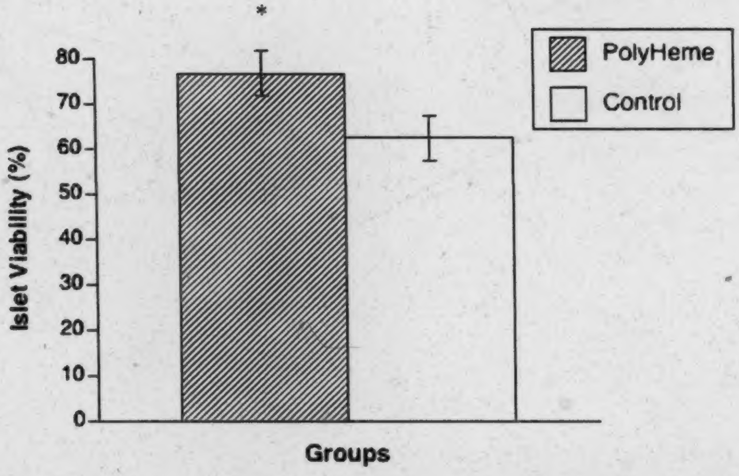

Figure 6-1. Percentage of viability in islets of pancreata from PolyHeme and Control groups.

There was no statistical difference in the viability of islets from the PolyHeme group (filled bar) and the control group (empty bar). Viability of islets from both groups was expressed in percentages of live vs. dead cells 24 hours after isolation. PolyHeme n= 9 isolations, Control n= 9 isolations. *p=0.04.

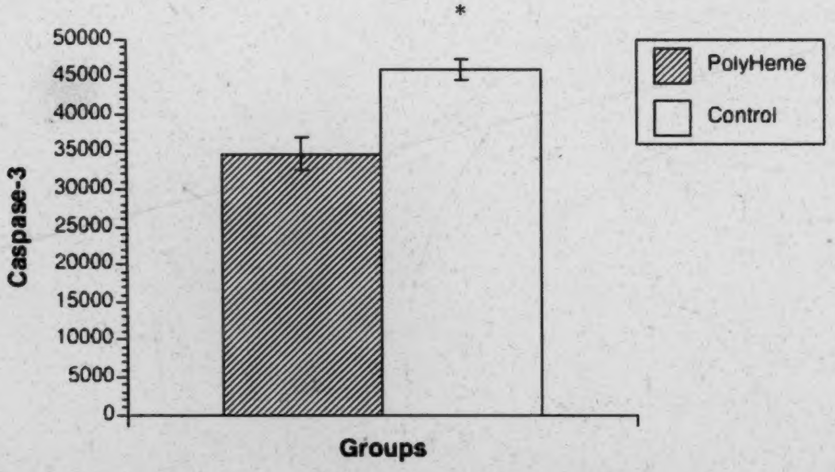

Figure 6-2. Caspase-3 levels in islets from PolyHeme and Control groups

The levels of caspase-3 were lower in islets from the PolyHeme group (filled bar) than the control group (empty bar) 24h after isolation n=3 isolations per group. *p=0.011.

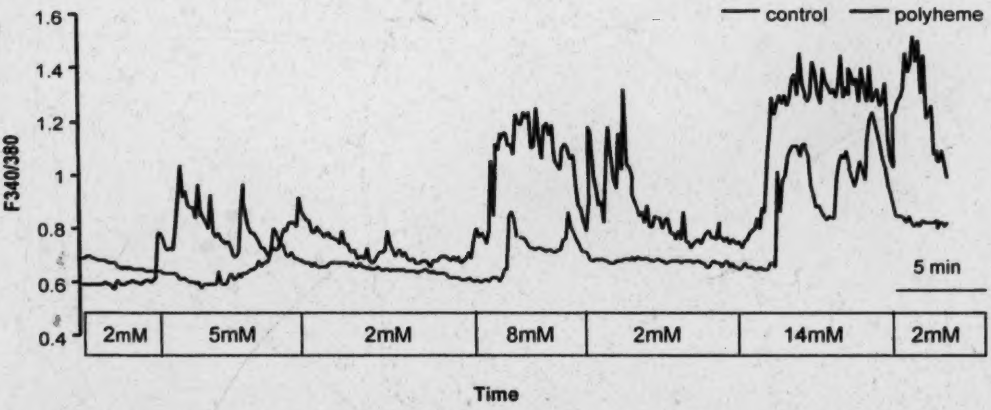

Figure 6-3A. Changes in intracellular calcium levels after a glucose challenge in "steps" in two representative islets from PolyHeme and Control groups.

Changes in ratio-metric values (Fura 2/AM) as a measurement of intracellular calcium levels in response to an increasing glucose challenge were more pronounced in islets from the PolyHeme group (red line) than for islets from the control group (blue line). Two representative islets (of 25) under basal (2mM) and stimulated (5, 8, 14mM) glucose conditions are depicted in this figure.

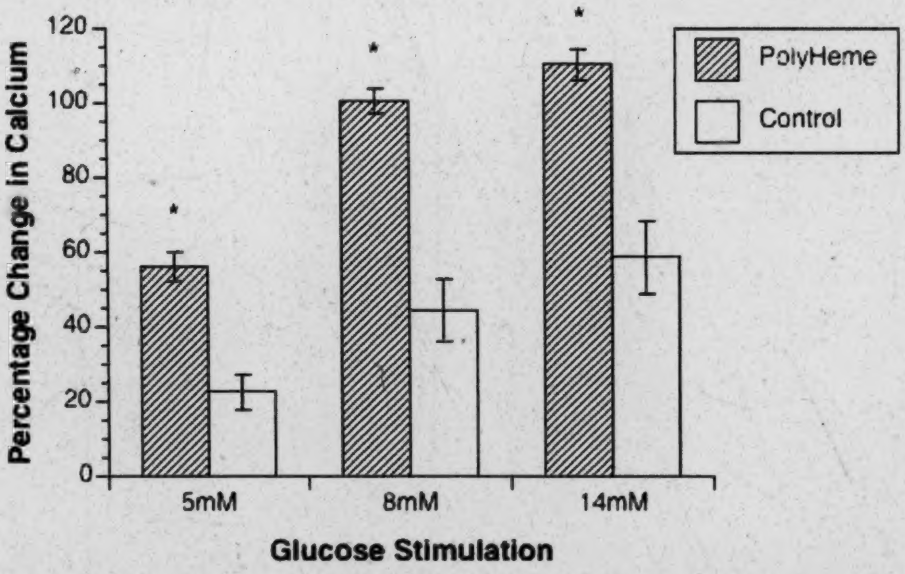

Figure 6-3B. Changes in the percentage of intracellular calcium in response to increasing glucose values in islets from PolyHeme and Control groups.

The percentage of change in intracellular calcium levels was higher for islets from the PolyHeme group than for islets from the control group in response to glucose stimulation (5, 8 and 14mM glucose concentrations), n=25 islets per group, p<0.05 at all glucose concentrations.

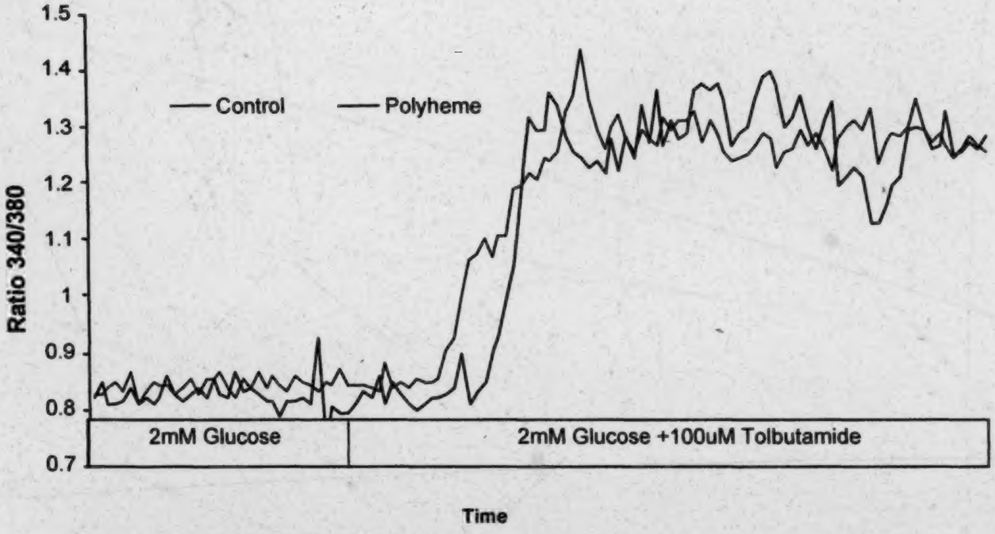

Figure 6-4A. Changes in intracellular calcium levels under basal conditions (2mM glucose) in two representative islets after addition of tolbutamide.

Changes in ratio-metric values (Fura 2/AM) as a measurement of intracellular calcium levels were similar in two representative islets (of 25) after the addition of Tolbutamide (100 µM) to the culture media.

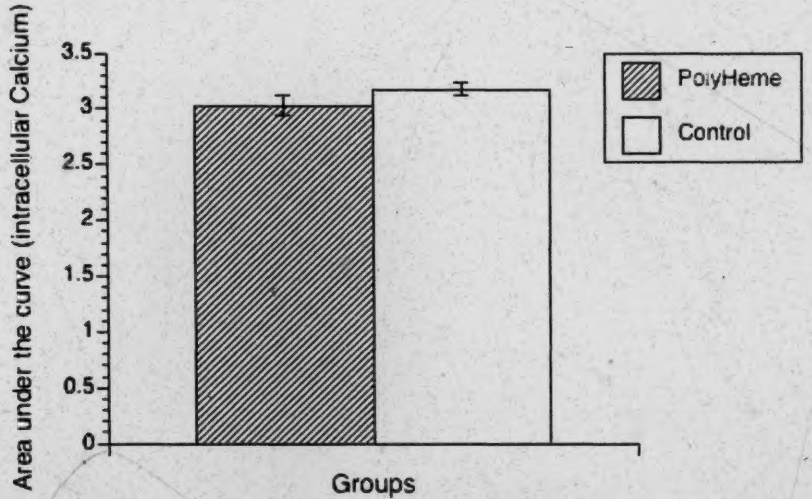

Figure 6-4B. Intracellular calcium levels in islet from PolyHeme and Control groups after addition of tolbutamide to the culture media.

The area under the curve (AUC) calculated for intracellular calcium levels under basal glucose concentration (2mM) in islets from PolyHeme (filled bar) and control (empty bar) were similar after the addition of Tolbutamide (100 µM); n=25 islets per group, p=0.183.

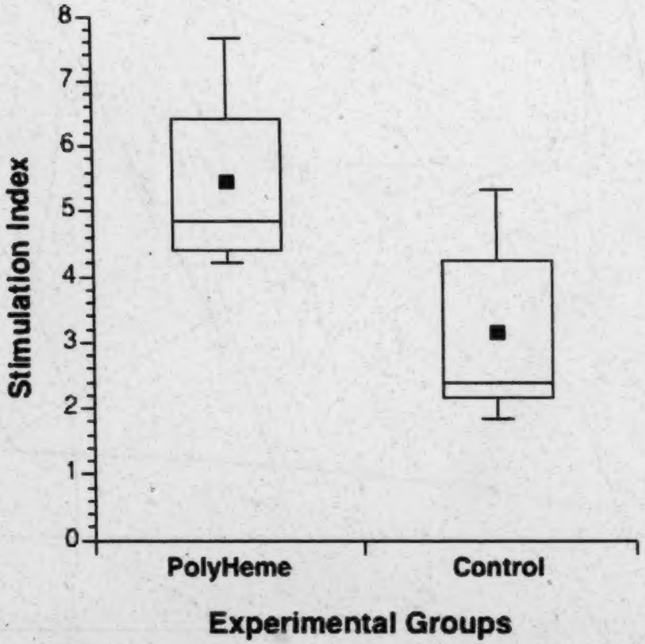

Figure 6-5. In vitro islet function assessment by Glucose Static Incubation in islets from PolyHeme and Control groups.

Insulin secretion of PolyHeme islets in response to a glucose challenge, (expressed as stimulation index, SI) was higher than secretion of insulin by islets from the control group. The black square dots represent the means, the middle line of the large square represents the median (50th percentile), the lower bar of the box represents the 25th percentile and the upper bar of the box represents the 75th percentile. PolyHeme n=5 isolations, control n=5 isolations. p=0.03.

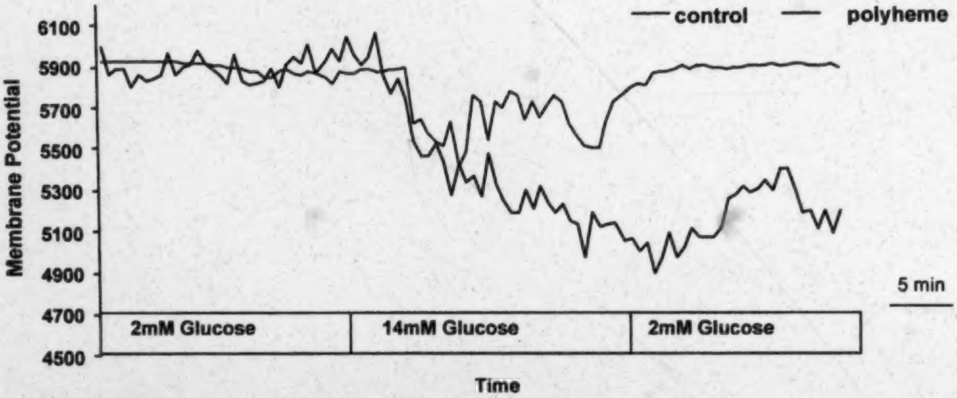

Figure 6-6A. Changes in mitochondrial membrane potential after glucose stimulation in two representative islets from PolyHeme and control groups.

The uptake of Rh123 into mitochondria after glucose stimulation was higher for islets from PolyHeme group (red) than for the control (blue). A gradual decrease in fluorescence represents the incorporation of Rh123 into the membrane as an indirect measurement of membrane potential. This represents an improved membrane potential for PolyHeme islets in response to glucose than the control islets. Two representative islets (of 25) under basal (2mM) and glucose-stimulated conditions (14mM) were used.

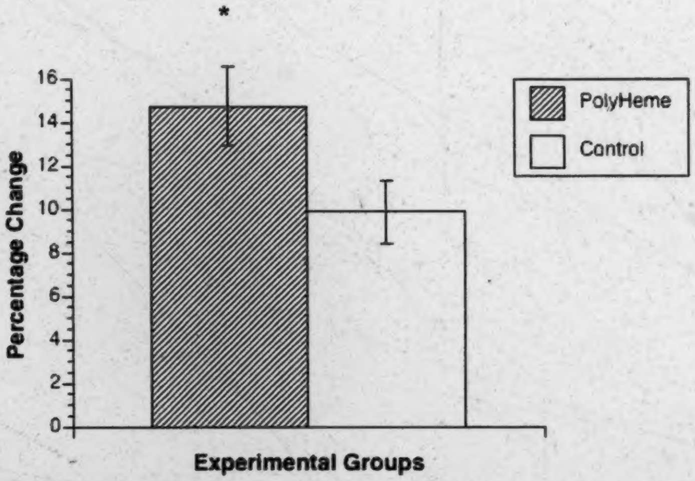

Figure 6-6B. Percentage change in mitochondrial potential after glucose stimulation in islets from PolyHeme and control groups.

The percentage of change in mitochondrial membrane potential after glucose stimulation was higher for islets from the PolyHeme group than islets from the control; n=25 islets per group, $p<0.05$.

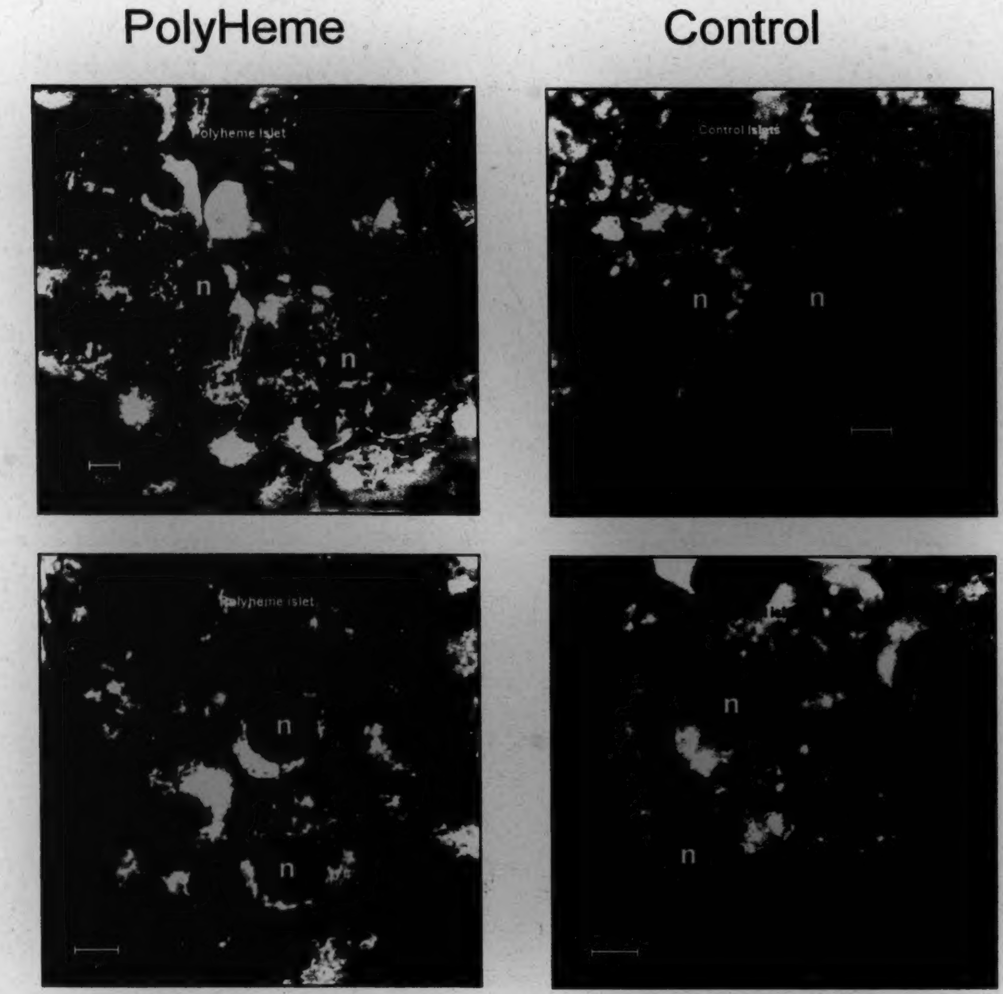

Figure 6-7. Mitochondrial morphology assessed by confocal microscopy in islets from PolyHeme and control groups.

Mitochondrial Morphology: Mitochondria were stained with rhodamine 123 dye. Two representative images (of 75) in individual islets from PolyHeme and control groups are shown. Images are maximum intensity projections, 1μm slice thickness. Cell nuclei in the islets are identified with the letter “n”. Mitochondrial morphology and distribution around the nuclei appear superior (increased fluorescence intensity) in the PolyHeme group than the control. Contrast has been balanced to reveal details of mitochondrial morphology. Scale bar is 5 μm.

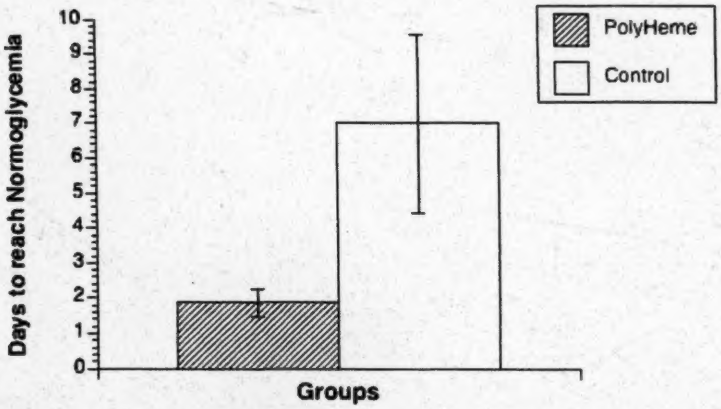

Figure 6-8. Number of days (lag time) to reach normoglycemia for mice transplanted with islets from PolyHeme and control groups.

The "lag time" to reach normoglycemia for mice transplanted with islets from the PolyHeme group (filled bar) was shorter (in days) than for mice transplanted with islets from the control group (empty bar). PolyHeme n=6 mice and control n=4 mice. *p=0.02.

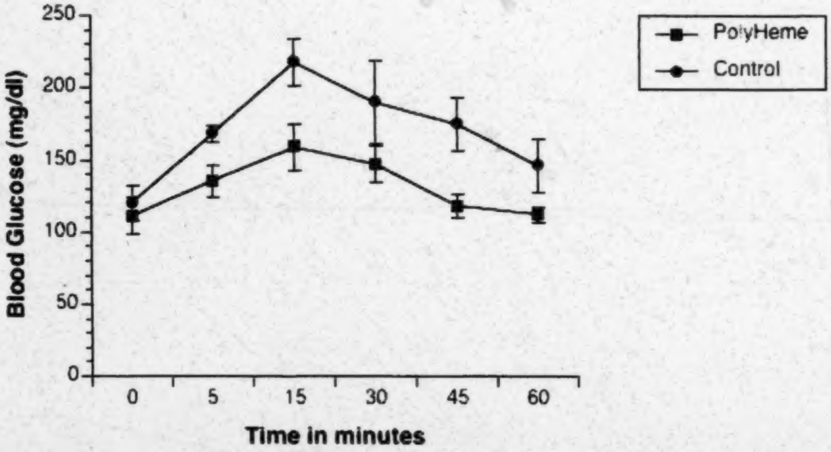

Figure 6-9. ***In vivo*** **function assessment through an Intraperitoneal Glucose/Arginine Tolerance Test (IPG/ATT).**

Mice transplanted with islets from the PolyHeme group (square dots) showed a normal response to a glucose/arginine stimulation test (lower blood glucose values), while mice transplanted with islets from the control group (circle dots) showed a diabetic response to the test. A representative sample of mice that reached normoglycemia after transplantation with islets from PolyHeme n=5 out of 6 and control n=3 out of 4. p=0.03.

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Chapter 7

Conclusions

Islet transplantation has proven to be difficult to master even in the best hands. It is dependent on the process of islet isolation, which is riddled with inconsistencies, where multiple factors potentially affect the final product, availability of islets. After the advent of new immunosuppression therapies and subsequent reduction in rejection rates, islet availability is one of the main obstacles for success in islet transplantation.

Pancreata for islet isolation are generally preserved with cold solutions to reduce the metabolic rate and energy requirement in order to decrease cell damage. This period of cold ischemia is kept as short as possible. However, with current organ procurement strategies, pancreata are still exposed to a variable period of warm ischemia. Warm ischemia is associated with cellular energy reserve depletion and ultimately oxidative stress.

This work has explored two strategies to reduce ischemia-induced cellular damage during islet processing. First, we thought of preventing oxidative injury by providing the pancreatic cell with an antioxidant precursor, glutamine. Second, we investigated reduction of ischemia by using a hemoglobin-based O_2 carrier. In our studies, the main pancreatic duct proved to be an effective and practical route of administration of the treatment therapies when injected with the collagenase enzyme during islet isolation.

The first study in rodents showed that 30 minutes of warm ischemia to the pancreas exposed islets to oxidative stress. It was also shown that adding 5mM L-glutamine to the intraductal enzyme perfusion improved islet isolation outcomes in terms of islet yield and cell viability. We also demonstrated that intraductal glutamine application effectively increased intracellular GSH levels. In our next study we showed that glutamine treatment with subsequent replenishment of GSH levels decreased oxidative injury and improved islet graft outcomes in terms of success rate and *in vivo* function in response to a glucose challenge.

In the following study, we examined the role of intraductal glutamine treatment in the clinical setting by using human pancreata from "marginal donors", which had been refused by other centers even for islet isolation. We found that glutamine perfusion improved *in vitro* and *in vivo* outcomes of the isolation. Glutamine treatment showed a decrease in the level of oxidative stress and oxidative injury to islets. Islet yield was increased and apoptosis was reduced in treated islets. Glutamine also improved islet graft outcomes, rendering a higher number of diabetic mice normoglycemic. Islet graft function was also improved shown by a better response to a glucose challenge in these mice.

Oxidative stress as a consequence of ischemia could be decreased by glutamine administration. In order to reduce ischemia and protect tissue from energy depletion and damage we further tested O_2 delivery to ischemic pancreata. In our last experiments we performed intraductal perfusion of rat pancreata with oxygenated human polymerized hemoglobin O_2 carrier (PolyHeme). Compared to the control (RPMI-1640), PolyHeme treatment improved islet isolation and transplantation results. To understand why O_2 delivery by PolyHeme improved islet quality we assessed the functional integrity of mitochondria. We observed higher intracellular Ca^{2+} levels in response to glucose stimulation and improved mitochondrial membrane potentials in PolyHeme treated islets.

We also found both, an improved glucose-stimulated insulin secretion and decreased cell death in PolyHeme treated islets. We tested the mitochondrial pathway by directly promoting insulin secretion through the addition of tolbutamide (a K^+-ATP channel inhibitor). Intracellular Ca^{2+} response to glucose resulted similar between both groups, suggesting that the provision of O_2 by PolyHeme protected the mitochondrial pathway in the process of insulin secretion in response to glucose. Mitochondria are the powerhouse of the cell and if damaged, can be a major contributor to apoptotic cell death. Improvement of *in vivo* function was also achieved in transplanted mice by a shorter lag time to reach normoglycemia and improved response to an intraperitoneal glucose stress test. In conclusion, the delivery of O_2 by PolyHeme reduced cell ischemia and did not increase oxidative stress.

Despite the tremendous advances that islet transplantation has made, cellular replacement therapy for the treatment of diabetes faces distinct challenges ahead, but where one could also envision opportunities for improvement. Recent improvements made in this field can be summarized and include the optimization of organ procurement techniques to reduce warm ischemia, organ storage through the routine use of PFC's and the TLM during pancreas transportation, more purified and stable enzyme blends to decrease the variability in pancreas digestion and islet isolation, the use of culture media supplements to improve islet survival in culture, the use of modified immunosuppressive therapies aimed to improve graft survival and function after transplantation, and the development of transplantation procedures that decrease the risks of the intraportal approach.

The Problem of Availability

Maybe the most immediate challenge in islet transplantation is finding an appropriate source of insulin producing cells. Currently, the only cells known to be capable of sensing human blood glucose levels and responding with appropriately regulated insulin secretion are the β-cells from the pancreatic islets of Langerhans. In this context, organ donation represents one of the most important aspects in the availability of islets for transplantation. Current donor rates in North America are approximately 18 pancreata per million population per year (approximately 316 million-US & Canada); this means only 5,700 pancreata available for transplantation. With an incidence of 30,000 new cases of type 1 diabetes per year in North America (plus approximately 1 million existing), this is only 0.57% of the need if one would want to provide therapy for every type 1 diabetic. One of the possible solutions to increase resources is the use of a non-heart-beating donor pool, which are not normally regarded as suitable for islet isolation, but as shown by the University of Pennsylvania group and a Japanese group from University of Kobe, they can be useful in successful single-donor islet transplants (1, 2). In contrast to non-heart-beating donors living donation could also potentially increase the donor pool (3), although there is still some controversy in this area, since it is difficult to justify the obvious disadvantages for the donor. Concomitantly, in order to relieve the shortage of donors, one of the challenges is to improve the efficiency of islet transplantation through achievement of insulin-independence with single donor grafts. Nevertheless, even with these strategies aimed to increase the donor pool and improve utilization of available organs, the shortfall in pancreas donation will probably make it necessary to find an alternate source of effective insulin-producing cells.

In this context, there are promising strategies that have been investigated to fulfill this task:

1) Multiplication of islets *in vitro*: A number of culture conditions have been studied for the propagation of β-cells or islets in vitro, but unfortunately non have been able to prevent the decline in insulin production by the cultured cells. In other words, no group had been able to reliably expand islet populations with robust insulin-producing capacity until quite recently, when a group in Japan described the establishment of a human pancreatic β-cell line through proliferation and immortalization of a purified β-cell with an oncogene that can be later deleted from the resulting cells, while still maintaining their insulin producing properties (4). This strategy holds the hope of potentially overcoming the limitation of primary pancreatic β-cells for transplantation and control of diabetes. However, I feel that further investigation is needed in order to study long-term effects and dissipate some of the safety concerns related to the use of oncogenes for cell proliferation.

2) Xenotransplantation: Pig islets; in particular have been considered as a non-perishable source of insulin-producing cells. Factors that advantage this strategy are for example that humans have been treated with porcine insulin for a number of years, pig islets regulate glucose in a similar range to human islets, they are relatively easy to breed, and since pigs are slaughtered for food consumption, it would be more ethically acceptable than providing islets from other species such as dogs. However, two main limitations for the use of pig islets are that firstly, humans express high titers of antibodies against a α-galacose residue that is present on most pig cells, resulting in a hyper-acute rejection. Secondly, pig cells contain endogenous retrovirus; there is the potential that they may infect the human host, especially under conditions of immunosuppression as is the case in transplantation. There are

currently strategies being investigated intended to prevent these situations. Some investigators in Cambridge, U.S. have created genetically modified pigs that do not express the galactose α- (1,3) galactose residue (5) and another group in Germany plans to breed these animals in special pathogen-free conditions to minimize risk of zoonotic disease (6).

3) β-Cell differentiation from stem cells: Embryonic stem cells are known to have pluripotent capacity. A research group has been successful in introducing the human insulin gene into mouse embryonic stem cells and selecting the insulin producing cells to treat diabetic mice (7), but it is difficult to translate into the context of human embryonic stem cells that grow more slowly and are more susceptible to death in culture than murine cells. If an *in vitro* process using human embryonic stem cells were successful, it would be of vital importance to eliminate every undifferentiated cell given to patients because of the risk that such cells might differentiate into teratomas. Research has been published using culture techniques promoting differentiation of stem cells into what appear to be insulin-producing cell clusters (8), but some at the University of New York have suggested that at least some of these anti-insulin antibody staining cell clusters may be artifactual, as a result of insulin uptake from the growth media, and that they lack the appropriate capacity for regulated insulin secretion (9). International study groups have produced data that has been difficult to analyze and draw conclusions from (7, 9). I feel that further studies are needed in order to expand upon these results. In any case, insulin-producing cells generated from progenitors *in vitro* will have to overcome tight regulations related to human safety before their widespread acceptance.

In the context of islet availability, pancreatic islet isolation, currently the most plausible of the approaches discussed, has experienced important advances. Research is focused on remaining challenges, some of which have been discussed in chapter 1 and will be revisited later in this chapter.

Loss of the β-Cell Mass.

Another important issue related to availability is islet cell loss post-transplantation. It is estimated that only 25-50% of the islet graft survives after transplantation, although others like Korsgren and collaborators estimate even lower values (10%-20%) (10). The possible explanations for the deterioration in islet mass over time have been suggested to include acute and chronic allograft rejection, local islet toxicity from immunosuppressive drugs, recurrent autoimmunity, and failure of the islets to regenerate over time. There is a true possibility that the loss of β-cell mass after transplant also may include donor-related circumstances. This may explain the discrepancy in the number of islets needed to achieve normoglycemia between allograft and autograft recipients. In this context, we should consider that the autograft pancreas has not been subjected to the disrupted physiology that accompanies brain death, while islets for clinical allogeneic transplantation come mainly from brain-dead donors. The presence of acute brain injury in the donor is associated with release of proinflammatory mediators (11). This "cytokine release" is highly toxic and directly inhibits glucose-stimulated insulin release. Some of the inflammatory cytokines released include interleukin-1α, interleukin-2, 6 and 10, which would be able to explain cases of primary non-function and acute allograft rejection. Another important aspect in islet cell loss at this stage could be related to hyperglycemia and hypoxia, which are important stress factors present at different points from procurement of the pancreas, throughout the isolation procedure, culture and transplantation. Hypoxia has been described also as a trigger for inflammation and secretion of pro-inflammatory cytokines. It has been proposed that the level of cytokines is inducible depending on events occurring to the islets before transplantation (12). These circumstances are capable of inducing or enhancing the immune rejection.

An important cause of early graft loss has recently been demonstrated through a thrombotic/inflammatory reaction occurring when islets come in direct contact with ABO-compatible blood (10). This is called instant blood-mediated inflammatory reaction (IBMIR) and results in activation of coagulation and complement systems. It can start through infiltration of islets by leukocytes as early as 15 minutes after intraportal injection. IBMIR is induced by pro-inflammatory cytokines, which as previously mentioned could themselves be induced by hypoxia. A group at University of Alabama has started to address this issue by pretreatment of the donor with anti-inflammatory and anti-apoptotic agents such as 17β-estradiol or atorvastatin, which have been shown to moderate the negative impact of islet damage induced by brain injury due to pro-inflammatory cytokines (11). Anti-thrombotic strategies to avoid IBMIR have also been developed through the use of compounds like nicotinimide, inactivated factor VIIa or low molecular weight dextran sulphate in order to inhibit tissue factor expression and cytokine release (13, 14).

There is though, another type of immune-mediated rejection, the clinical pattern of islet allograft rejection is acute allorejection. Allogeneic antigens class I and II [part of the major histocompatibility complex (MHC)] promote T-cells to mediate an immune response. The objective of preventing T-cell mediated allograft rejection is to target clonal activation and deletion by creation of tolerance without the need of non-selective T-cell depletion. The objective of tolerance in islet transplantation would mean a durable survival of MHC-mismatched islet allografts without maintenance immunosuppressive therapy and without rejection or loss of functional islet mass or insulin secretory reserve.

The immunosuppression therapy described by the Edmonton group is one of the most important developments in the clinical success of the Edmonton Protocol (15). The basis for this success was the avoidance of steroid immunosuppressive compounds, which are diabetogenic and result in a "burn out" of the islet graft.

Although the new therapy seemed to confer protection against allograft rejection and autoimmune recurrence, it remains quite broad in targeting the T-cell pathway of the immune response. This maintains a small, but increased risk in the development of malignancies that make a non-life saving transplant procedure harder to justify in patients with less compromised forms of diabetes. In addition, the compounds have a series of side effects that include pneumonitis, suppression of bone marrow function, mouth ulcers, deteriorating renal function, hyperlipidemia, hypertension, weight loss and fatigue, that have made the quest for less toxic compounds an immediate challenge in islet transplantation. The development of more T-cell specific or tolerogenic therapies to my view should sought after.

Induction of Tolerance

In this context, one strategy studied is the blockade of costimulatory molecules on the surface of T-cells, which interferes with their activation and clonal expansion, leading them to apoptosis. In my opinion, this is one of the most promising approaches currently tested. Interesting work has advanced in Emory and Edmonton with the use of a potent costimulatory signal blocker of CD28, "LEA29Y" that inhibits activation of allogeneic T-cells without massive destruction of the existing population, and has shown improved islet survival in primates (16). In addition, Wang and collaborators have demonstrated that blockade of CD28/B7 and OX40/OX40L co-stimulatory signal pathways prolongs the survival of islet xenografts in a model of rat islets into BALB/c mice (17). In another study, blockade of CD154 costimulation with hu5C8, a specific monoclonal antibody was shown to induce long-term insulin independence after islet transplantation in a primate model (18). Other studies that have been performed in primate models include an anti-CD3 diphtheria-based immunotoxin, which has shown to regulate long-term survival of islet xenografts and allografts (19). Another agent, FTY720 that causes redistribution of lymphocytes from circulation to secondary lymphoid tissues has proven effective in regulating autoimmunity (20). Moreover, FTY720, when used together with Cyclosporin A, a calcineurin inhibitor, was able to reduce xenograft rejection in a pig to rat model of islet transplantation (21). A T-cell depleting antibody alemtuzumab has shown to enhance islet survival post-transplantation through the reduction of immune sensitization, although in a recent Edmonton study which included a small number of patients this agent did not show improved outcomes compared to the current standard therapy (22). In terms of achieving tolerance, induction of "mixed chimerism" has been performed through donor-specific bone marrow transplantation (pre-conditioning therapy). This strategy has proven successful for some solid organ transplants, but will surely have to be further scrutinized before applying to clinical islet transplantation, especially due to the risk of graft vs. host disease, which can cause significant morbidity.

In the case of islets, prevention of immune rejection can be promoted both by immunosuppression therapy and by the establishment of physical or mechanical barriers between islets and immune cells. In order to prevent T-cells from coming into contact with the graft resulting in cell mediated rejection, the islets can be immunoisolated by encapsulation.

Islet Encapsulation

Islets are encapsulated with a semipermeable membrane so that cells can be protected from the host's immune system while still able to exchange nutrients and cell secretions across the membrane. Encapsulation of islets would potentially eliminate the requirement for immunosuppressive drugs. Several biomaterials have been tested for these devices, of which probably alginate has been the most successful. Around the alginate core, poly-L-lysine constitutes a semipermeable membrane that can be modified to achieve different degrees of permeability. Clinical application of this strategy has been difficult to establish and the main concerns include inadequate supply of oxygen to islets inside the capsule, foreign body reaction of the organism against the capsule materials, quality control, inability of producing a uniformly successful microcapsule, non-specific immune-mediated response and specific indirect rejection mechanisms resulting in graft loss. Problems can be summarized in poor tolerance of islets to the microenvironment, biocompatibility problems and inability of islets to select permeability to inflammatory cytokines. Nevertheless, with development of new encapsulation techniques, improvement in the purity of the materials (alginates) and improvement in oxygen supply or hypoxia resistance, transplantation of encapsulated islets has become a clinical reality. One of the current hurdles is still the site of implantation, since the capsules increase the mass of tissue for transplantation and generally require an intraperitoneal implantation, which further compromises blood supply and oxygenation to the islets. Nevertheless, encapsulation promises to potentially avoid the problem of patient sensitization and theoretically as many transplants as required may be performed in order to achieve insulin-independence. A few patients have been transplanted in the U.S. with variable results and currently in Europe (Italy), clinical trials are underway using the peritoneum as the site of implantation. I think that results will be mostly dependent on the quality of materials (alginates) and the encapsulation technique, but it remains to be seen the efficacy and long-term function of these grafts to achieve and maintain normoglycemia.

The problem of long-term survival

Considering the issues discussed above, islet transplantation has been successful in achieving insulin independence and relieving problems with hypoglycemia.

However, if we base our observations in the Edmonton trial, we see that the rate of insulin independence 1 year post-transplant was 80%, at 3 years it decreased to 50%, and currently at 5 years it is 10% with most of the patients returning to some use of exogenous insulin. Although most of these patients still have some degree of endogenous insulin secretion (80% C-peptide positivity), there is clearly an inadequate insulin reserve, maybe due to loss of islet mass or loss of function over time. According to Dr. Ryan and colleagues from Edmonton, acute insulin response to secretagogues is well below normal even shortly after transplantation in these patients, suggesting that the islet mass at that early point is probably marginal. Moreover, even with excellent function, the fact that usually fasting glucose values were higher than normal, may reflect some problem with either islet mass or function from the beginning.

The discrepancy between short and long-term post-transplantation outcomes may have a multifactorial source, ranging from acute islet mass loss due to inflammatory reactions to allo- or autoimmune rejection. There is also a possibility that islets are being overstressed by the development of insulin resistance in these patients. In addition, one or more of the immunosuppressive drugs used in the protocol have some degree of direct toxicity to the islets, as for example it has been shown in rodent models that Rapamycin (Sirolimus), with its antiproliferative properties, is also associated with insulin resistance (23) and when combined with FK506 (Tacrolimus), which on its own can decrease β-cell function, may be able to induce diabetes. Glucose control after islet transplantation deteriorates with higher doses of the immunosuppressive drugs (24), a problem which is exacerbated by the lack of markers of rejection for islets that makes the adjustment of doses a challenge of its own. Another problem related to the drugs is that by using the hepatic location for implantation, islets are also exposed to the first pass of immunosuppressive drugs, potentially increasing the risk of dysfunction due to toxicity. Another reason for loss of function over time could be that blood supply to the islet graft at the hepatic site does not develop following the anatomical pattern of the islets in the pancreas (from the center out), this may be affecting the normal function of specially β-cells, which are located towards the center of the islet. Moreover, the liver site of implantation may be also interfering with islet function from the standpoint of metabolism in that islets are exposed to high concentrations of nutrients through the portal vein, and insulin release may be directly into the hepatic vein.

Clearly, one cannot easily dissect each possible factor responsible for the loss of insulin-independence and islet function over time, but as the problems associated after islet transplantation become more explicit, current and future research may hold some of the answers to these questions in the short run.

Where would intervention make a difference?

The herein presented work indicates that both reduction of ischemia by the use of an O_2 carrier and reduction of oxidative injury by applying an antioxidant precursor can improve outcomes of pancreatic islet isolation. Future work should be directed to further reduce ischemia by improving O_2 delivery during critical periods of warm ischemia, as during the procurement, digestion and transplantation. Summarized in Figure 7-1 is an example of where in the process there is potential for warm ischemia-mediated injury to pancreatic tissue and islets. In this context, the presented work should project into other studies that can investigate alternative strategies targeting these critical areas.

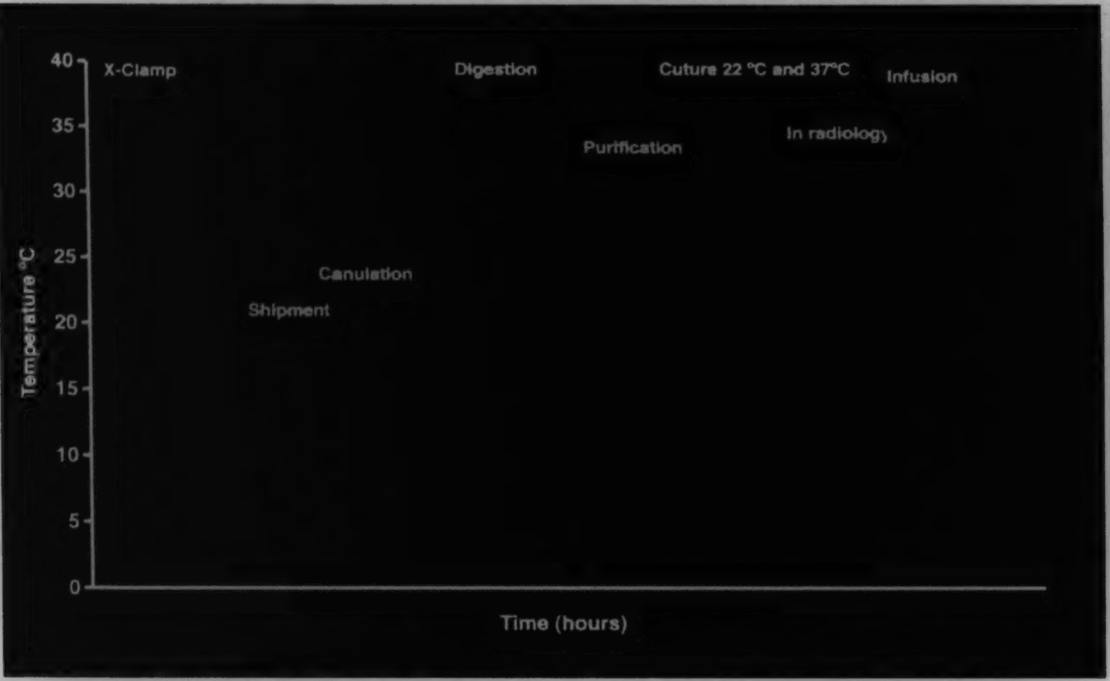

Figure 7-1. Areas of warm and cold ischemia with potential injury to pancreatic tissue and islets.

The graph depicts the different temperatures to which pancreatic tissue and islets are exposed throughout the whole process from procurement to islet transplantation. Red circles indicate areas of warm ischemia were oxygenation and antioxidative therapy would be most beneficial. (Avila J, Shapiro AMJ, Oberholzer J, Lakey JRT, Anderson C, Rajotte RV, and Churchill T. As generated during the discussion of this dissertation, Surgical-Medical Research Institute, January/2006).

Starting with an organ donor, the cause of death and the circumstances around it may already decide the fate of the organs for transplantation in terms of warm ischemia, depending essentially on the maintenance of an adequate blood supply to the organs (maintenance of cardiac activity). In the case of unknown "downtime", there are not many variables that we can control and I will not enter into detail with a proposed intervention at this level. Nevertheless, it may be worth of notice, as we mentioned previously, that there are groups working on the optimization of techniques leading to the use of non-heart beating donors for islet isolation, although under these conditions certain variables are still controlled. The implementation of special measures is performed in order to prepare the donor for procurement surgery immediately after the cessation of cardiac activity with minimal amount of warm ischemia. Groups at University of Pennsylvania in the U.S. and University of Kyoto in Japan have introduced special techniques like the use of an *in situ* regional organ cooling system originally developed for kidney procurement, but which has allowed Japanese surgeons to make use of pancreata from non-heart beating donors resulting in successful islet isolation and transplantation (2, 25). I regard this kind of intervention to be at the level of organ procurement. Here, strategies should not only include techniques related to the administration of cold solutions, but also solutions that promote the recovery of tissue once a period of warm ischemia is inevitable. Solutions containing multivitamins, with aminoacids and antioxidants that help the tissue recover from stress related to warm ischemia. In this context, I propose the investigation of the effects of oxygenated Polyheme/glutamine administrated intravascularly immediately after cessation of cardiac activity on ischemic cell damage in a rat model of warm ischemia. Depending on results, and once ethical approval is gained; this therapy would be applicable in the procurement for multiorgan recovery. I think that minimizing warm ischemia at this level and optimizing procurement techniques, already by themselves would be able to produce improved outcomes during islet isolation. Advances in this area have been shown previously by Lakey and collaborators in Edmonton by efficient in situ cold flush and maintenance of a low core temperature of the pancreas during procurement (26). Also, the Miami group optimized pancreas procurement through careful and meticulous dissection of the pancreas and its vasculature by an experienced surgeon, resulting in higher success rate of the isolation procedure (27).

Taking just one step back, as mentioned above, even with uninterrupted cardiac activity, brain death produces a series of changes that can rapidly lead to the hemodynamic instability of the patient with direct impact on the organs for transplantation. I would propose the implementation of strategies at this level to prevent the release of pro-inflammatory cytokines, like those adopted by Contreras and collaborators with the use of 17β-estradiol or atorvastatin (28, 29). I would propose the addition of oxygenated solutions like PolyHeme/glutamine or other sources of oxygenation to the patient in order to minimize hypoxia to the organs, which is also known to promote the production of pro-inflammatory cytokines.

During cold storage and shipment of the pancreas ischemia can cause deleterious effects, although at this level the cold temperature delays the development of ischemic injury. Nevertheless, hypoxia is still an obvious event at this stage, which should be counteracted with the implementation of O_2 or oxygenated solutions, despite the low temperatures. One of the most extraordinary advances in order to prevent ischemic injury at this level is the implementation of the TLM cold storage using PFC's and UW solution, which has been shown to reverse the damaging effects of warm ischemia, increase islet yields and improve engraftment. The idea of an intravascular organ flush in the back table with oxygenated solutions is abrogated before packaging of the organ and shipment. In this context, I propose the use of mechanical devices (pulsatile pumps), which have already been successfully implemented for the transport of kidneys for transplantation. This pump would theoretically maintain a cardiac-like intravascular flush of solutions (namely PolyHeme) in order to prevent hypoxia and maintain an appropriate tissue perfusion. Although the concept sounds quite promising, this strategy has been tried previously in Edmonton with less than encouraging results, suggesting the need for further and more detailed research in this field.

Once at the processing facility, there is ample space for improvement of current isolation techniques. Work has already started in order to evaluate islet isolation and transplantation outcomes after intraductal administration of a hemoglobin oxygen carrier and L-glutamine in human pancreata procured from cadaveric donors. The intraductal perfusion of the pancreas with preservation solutions has been adopted in the past for its practicability for enzyme delivery and the potential for protection of the organ. In this sense, UW solution has been used with favorable results in terms of islet yields. However, I feel that we could take advantage of this route by the administration of oxygenated solutions, of which PolyHeme seems appropriate due to its solubility and based on the presented results in this thesis, when investigated in the rodent model. Another solution that may complement the administration of PolyHeme at this point is N-acetyl cysteine, a cysteine derivative that is widely used as an antioxidant and may have similar or superior antioxidant properties than glutamine when used in cells under cold ischemia.

The digestion step of the isolation is probably one of the most critical events of the isolation procedure, where the islets are exposed to the activity of the collagenase enzyme at warm temperatures (37ºC). At this level, important advances have been achieved in relation to the purity and stability of the enzyme blends used, enhancing the reliability of the isolation. A new enzyme preparation composed of a highly purified collagenase that can be reproducibly blended with predetermined amounts of neutral protease has been recently evaluated with encouraging results (30). The absence of lot-to-lot variability in terms of neutral protease to collagenase ratio has made this new enzyme promising for human islet isolation. In addition, islets are faced with warm ischemia during this period, where my proposed intervention could probably make the most difference. The prevention of warm ischemia may result a challenge since the temperature for the optimal enzyme activity is 37º C. The availability of O_2 at this point may prove essential for the improvement of islet survival, viability and function. One could also study the effects of adding PolyHeme/glutamine and continuous O_2 flow (100%) to the digestion. This strategy would assure O_2 delivery and antioxidant treatment to the islets during this critical step.

Following the isolation procedure, now that a culture period of islets before transplantation is acquiring more importance among different groups, and since at this point islets are still faced with warm ischemic insult, the proposed concept would also be appropriate and applicable at this stage. Islets being formed by a compound of cells, O_2 diffusion to the more centrally located ones (usually β-cells) is greatly compromised in culture. It would be fascinating to study the effects of O_2 delivery by PolyHeme/glutamine in islet cell culture immediately after the isolation procedure, especially on islets incubated at different O_2 concentrations, and then assess islet function and viability. With the advent of new cell culture devices like rotating wall vessel type bioreactors, islets can be transported even through long distances with minimal physical injury. This concept can be combined with PolyHeme by using a PolyHeme/glutamine based culture media and provide islets with physical and antioxidative protection, in order to transport islets in culture for transplantation at remote centers from the isolation facility. The preculture period provides opportunities for modifying pretransplant conditions in the recipient (e.g. immunosuppressive induction), and manipulation of the graft in order to improve survival and function after transplantation. Important advances are currently being made with the use of antithrombotic strategies during culture (31). These include the use of nicotinamide, inactivated factor VIIa or low molecular weight dextran sulphate developed by Korsgren and collaborators in order to prevent IBMIR (13, 14). This period provides an excellent opportunity to try different O_2 delivery strategies, even by simply modifying O_2 conditions in the incubator where islets are kept. In addition, the use of proliferation factors and anti-apoptotic compounds during this time may also increase graft survival and function.

Moving along into the transplantation, we have previously discussed about the different mechanisms potentially involved in islet cell loss during this phase. In order to summarize, graft injury and loss may be triggered as soon as 15 minutes after intraportal infusion through the activation of IBMIR, mediated through the release of proinflammatory cytokines. Islet cell loss could also happen after transplantation through acute allo- or autoimmune rejection, direct drug toxicity, metabolic stress after development of insulin resistance or presence of toxins at the hepatic site, abnormal re-innervation or re-vascularization of the islets, and the activation of apoptosis from oxidative stress and reperfusion injury. Any of the strategies developed to target one or more of these issues, in my view can produce important improvements in islet graft survival and function over a longer term than currently observed.

I feel that my contribution during this step is by developing an oxygenated solution were the islets would be transplanted into the portal system, but without the state of hypoxia in which they are transplanted according to current protocols. Hence, some inflammatory reactions could be prevented and islets speared from initial injury that leads to apoptosis. In order to achieve this I propose transplanting islets in oxygenated PolyHeme/glutamine transplant media and evaluating transplantation outcomes in animal models after intraportal infusion. The animals would be given 100% O_2 throughout the transplant procedure in order to increase O_2 concentration in intraportal circulation. A more clinical application in the short term would be administrating 100% O_2 free flow to the patient in the radiology department during the transplant, which would probably not require ethical approval an would be relatively simple to achieve. I also believe in the manipulation of islets with strategies aimed at reduction of apoptosis before transplantation, in which I would like to emphasize. An example is described by Emamaullee and collaborators, where islets were adenovirally transfected in order to express an anti-apoptotic protein, which protects islets from apoptosis after transplantation, period in which islets are known to mostly succumb to apoptosis (32). By using this strategy the investigators achieved a more rapid reversal of diabetes in animals transplanted with islets expressing the anti-apoptotic protein (XIAP), and enhanced resistance of islets to oxidative injury produced *in vitro*.

Providing that these strategies produce the results expected, the optimization of each of these steps would increase efficiency throughout the whole process. The contribution of this therapy is aimed to increase islet supply for transplantation and the preservation of long-term function of islet grafts through the improvement of pancreas preservation, isolation and transplantation techniques.

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